AD_____

GRANT NUMBER DAMD17-94-J-4306

TITLE: Cell-Matrix Interactions in Breast Carcinoma Invasion

PRINCIPAL INVESTIGATOR: Anna M. Curatola, Ph.D.

CONTRACTING ORGANIZATION: New York University Medical Center
New York, New York 10010-2598

REPORT DATE: January 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1999	Annual (15 Dec 97 - 14 Dec 98)

4. TITLE AND SUBTITLE

Cell-Matrix Interactions in Breast Carcinoma Invasion

5. FUNDING NUMBERS

DAMD17-94-J-4306

6. AUTHOR(S)

Curatola, Anna M., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

New York University Medical Center
New York, New York 10010-2598

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT

The α6β4 integrin is a laminin receptor expressed on the basal, basement membrane-apposed surface of ductal breast epithelial cells. In contrast to all other known integrins, α6β4 is concentrated in hemidesmosomes, adhesive junctions which connect the basement membrane to the intracellular keratin cytoskeleton. In virtually all cases of human breast cancer analyzed, α6β4 has been found to be diffusely distributed at the cell surface instead of being concentrated in hemidesmosomes. Our previous studies have indicated that α6β4 promotes the assembly of hemidesmosomes by interacting, via a specific region of the large unique cytoplasmic domain of the β4 subunit, with cytoskeletal elements of hemidesmosomes. We have observed that ligation of the EGF-R or EGF-R/Neu heterodimer promotes the association of the Src-family kinase Fyn with α6β4 and mapped the sequences of Fyn and β4 required for the interaction. Ligation of the EGF-R or EGF-R/Neu heterodimer causes tyrosine phosphorylation of the β4 tail and disassembly of hemidesmosomes. This phenomenon appears to be mediated by Fyn because a dominant negative version of the kinase inhibit tyrosine phosphorylation of the β4 tail and disassembly of hemidesmosomes. Introduction of dominant negative Fyn also significantly inhibited EGF-promoted tumor cell invasion through matrigel.. These observations indicate that overexpression of the EGF-R and/or Neu facilitates tumor invasion by causing disassembly of hemidesmosomes.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	108
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Anna Marie Carabello 1/11/99

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Cell-matrix interactions are likely to play an important role in breast tumorigenesis. Most human breast cancers arise from the transformation of ductal epithelial cells (Wellings et al., 1975; Dairkee et al., 1985; Rudland, 1987). Normal ductal epithelial cells rest on a basement membrane, to which they adhere tightly (Dairkee et al., 1985). The adhesion of normal breast epithelial cells to the basement membrane is thought to be important for the organization of the cytoskeleton and the consequent establishment of polarity. In addition, normal breast epithelial cells receive signals from the basement membrane and these signals help them maintain a differentiated phenotype (Streuli et al., 1991). When compared to normal cells, breast carcinoma cells show a defective interaction with the basement membrane. First, like most carcinoma cells, they fail to assemble basement membrane components in an organized extracellular matrix, both *in vivo* and *in vitro* (Dulbecco et al., 1988; Petersen et al., 1992), and show cytoskeletal defects (Trask et al., 1990). Second, in contrast to normal breast epithelial cells, carcinoma cells do not arrest their growth when placed in a reconstituted basement membrane gel (Petersen et al., 1992). It is important to understand the molecular basis of these phenomena because they are likely to contribute to the ability of breast carcinoma cells to detach from the original tumor and invade adjacent tissues.

The molecular characterization of integrins provides a unique opportunity to examine the role of cell-matrix interactions in breast cancer. The integrins are a large family of adhesion receptors which bind to extracellular matrix components and, in some cases, to counter-receptors on other cells (Hynes, 1992). They consists of two distinct membrane-spanning subunits, α and β. At present we know of at least 9 homologous β subunits and 15 α subunits which can combine to form 21 receptors with distinct ligand binding specificities. Both the α and the β subunit (each ca. 140-200 kD m.w.) have a large extracellular portion, a transmembrane segment, and a short cytoplasmic domain. A notable exception is the $\beta 4$ subunit that has a large cytoplasmic domain. While the extracellular N-termini of α and β subunits associate to form the ligand binding pocket, the cytoplasmic domains of integrins interact with intracellular molecules.

The binding of integrins to extracellular matrix components promotes cell adhesion or migration, but ligation of integrins also results in intracellular signals which influence proliferation and differentiation (Giancotti, 1997). While contact with extracellular matrix components is required for the progression of normal cells through the cell cycle, a phenomenon called anchorage dependence, strong adhesion to an organized extracellular matrix seems to be able to limit cell proliferation (Giancotti and Ruoslahti, 1990) and promote differentiation (Streuli et al., 1991). The ability of integrins to modulate gene expression may help to explain the effects that the extracellular matrix has on proliferation and differentiation. The mechanisms by which integrins affect gene regulation are not completely understood, but likely depend on the ability of the cytoplasmic domains of integrins to interact both with the cytoskeleton (Burridge et al, 1988) and with

signaling molecules, such as the adaptor protein Shc (Mainiero et al., 1995; 1997; Wary et al., 1996) and Focal Adhesion Kinase (FAK) (Burridge et al., 1992).

Neoplastic cells are characterized by a number of adhesion abnormalities which may explain their ability to grow independently of the positive and negative control signals originating from the extracellular matrix (Giancotti and Mainiero, 1994). Virally transformed fibroblasts have a more rounded morphology in culture than their non-transformed counterparts. In addition, they often lack a cell surface fibronectin-containing pericellular matrix (Ruoslahti, 1984). The defective fibronectin matrix of transformed fibroblasts may only partially be attributed to either decreased biosynthesis or increased proteolytic degradation of fibronectin, since the fibronectin secreted by transformed cells is regularly incorporated in the extracellular matrix by normal cells (Hayman et al., 1981). This suggests that transformed cells can not retain at their surface the fibronectin they produce, perhaps because of a defect in the integrin receptors. Several observations indicate that the expression and function of integrins are altered in neoplastic fibroblasts. While in normal fibroblasts the β1 integrins, which include the α5β1 fibronectin receptor, are clustered in focal adhesions (Chen et al., 1985; Damsky et al., 1985; Giancotti et al., 1986), transformed fibroblasts lack such structures and their β1 integrins are found diffusely distributed over the cell surface (Giancotti et al., 1986; Chen et al., 1986). In addition, in fibroblasts transformed by tyrosine kinase oncogenes the β1 subunit is found to be partially phosphorylated on a tyrosine residue (Hirst et al., 1986), a phenomenon which may reduce its ability to interact with the cytoskeleton (Tapley et al., 1989). Finally, the expression of α5β1 and of another β1 integrin, probably α1β1, is suppressed in fibroblasts transformed by oncogenic viruses (Plantefaber and Hynes, 1989).

We have tested the hypothesis that changes in the level of expression or function of the α5β1 fibronectin receptor contribute to the adhesive abnormalities of transformed fibroblasts by overexpressing this integrin in Chinese hamster ovary (CHO) cells (Giancotti and Ruoslahti, 1990). The CHO cells have a transformed morphology, deposit little fibronectin in their pericellular matrix and are tumorigenic in vivo. As a result of the α5β1 overexpression, the CHO cells accumulated a fibronectin matrix and became less migratory. These results indicate an inverse correlation between matrix assembly of fibronectin and cell migration and suggest that the loss of fibronectin matrix and the increased invasive ability of transformed fibroblasts can be both brought about by a reduced expression or function of α5β1. Interestingly, the CHO cells overexpressing α5β1 were also found to be more anchorage dependent than the controls and were not able to form subcutaneous tumors in nude mice. K562 leukemia cells selected for high level expression of α5β1 show a similar normalization of growth properties (Symington, 1990). Conversely, CHO cells selected for their low levels of α5β1 expression are more tumorigenic than unselected cells (Schreiner et al., 1991). Thus, it appears that changes in the level of expression or activity of certain integrins may not only be responsible for the adhesive defects of neoplastic cells but may also contribute to their unregulated growth. Taken together, these observations suggest that the role

of integrins in tumorigenesis is twofold: first, integrins mediate stable adhesion or migration onto extracellular matrix components and changes in their level of expression and function may, therefore, contribute to tumor invasion. Second, integrins transmit signals from the extracellular matrix to the cell interior and these signals affect cellular growth and differentiation. Therefore changes in integrins may contribute to the unrestrained growth and lack of differentiation of neoplastic cells.

Although the adhesive phenotype of breast carcinoma cells is less well known that that of neoplastic fibroblasts, certain rules learned from the analysis of virally transformed fibroblasts seem to also apply to these cells. For example, breast carcinoma cells fail to assemble basement membrane components in an organized extracellular matrix (Dulbecco et al., 1988; Petersen et al., 1992) and show enhanced ability to grow when confronted with a reconstituted basement membrane gel (Petersen et al., 1992). Immunohistochemical studies have indicated that the expression levels of the $\alpha 2\beta 1$ collagen/laminin receptor, the $\alpha 5\beta 1$ fibronectin receptor and the $\alpha 6\beta 4$ integrin are altered in human carcinomas of the breast (Zutter et al., 1990; Koukoulis et al., 1991; Natali et al., 1992). In addition, while integrins are generally polarized at the basal or baso-lateral surface in normal breast epithelium, the integrins expressed in breast carcinoma cells are diffusely distributed over the cell surface (Zutter et al., 1990; Koukoulis et al., 1991; Natali et al., 1992). It is our hypothesis that these phenomena contribute to the ability of breast carcinoma cells to detach from the original tumor and invade the adjacent tissues.

BODY

We have initially focused on establishing a transgenic mouse model system in which to investigate the role of integrin defects in breast cancer progression. To this end, we have examined transgenic mice carrying either an activated or a normal form of the N-Ras oncogene under the control of the Mammary Tumor Virus Long Terminal Repeat (MMTV-LTR) promoter. These mice, similarly to mice carrying activated forms of the H-Ras or Neu oncogenes, develop mammary carcinomas with a high frequency during the first few months of their life (Sinn et al., 1987; Muller et al., 1988; Mangues et al., 1992; R. Mangues & A. Pellicer, Department of Pathology, N.Y.U. School of Medicine, unpublished results). The tumors which develop often consist of areas of different level of histological differentiation and thus can provide an insight to the process of primary breast tumor progression.

Immunohistochemical analyses conducted on these tumors revealed a significant loss of laminin staining. The $\alpha 6$ and $\beta 4$ subunits were not downregulated, but lacked polarization; some basal staining could be seen in better differentiated tumor areas. The $\beta 1$ staining was similarly no longer polarized. The $\alpha 2$ and $\alpha 3$ subunits were also diffusely distributed at the tumor cell surface with an apparent increased intensity of $\alpha 2$ staining. The $\alpha 5$ and αv subunits were not expressed in the tumors. To determine if the changes in integrin expression *in vivo* were a direct

result of Ras or due to other genetic changes which occur during tumor progression, the effect of the expression of the N-Ras oncogene on integrin expression in a normal murine breast cell line was investigated. The results indicated that Ras and ERb2/Neu cannot directly affect integrin expression and suggested that the changes in integrin expression observed in the transgenic model were not a direct consequence of oncogene action, but were caused by additional genetic changes associated with tumor progression.

Considering the altered cell surface distribution of α6β4 observed by us in the mammary tumors of transgenic mice and by others in human breast carcinomas, we decided to focus on examining the mechanisms by which neoplastic transformation could alter the subcellular distribution of this integrin.

Association of α6β4 with cytoskeletal and signaling molecules

In contrast to all the other known α and β subunit cytoplasmic domains, which are relatively short, the intracellular portion of β4 subunit measures over 1000 amino acids in length and contains, in its C-terminal half, two pairs of type III fibronectin-like repeats separated by a 142 amino acid Connecting Segment (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). While β1 and αv-subunit containing integrins interact with the actin cytoskeleton and localize to focal adhesions, α6β4 is found concentrated at hemidesmosomes both in cultured cells and in vivo (Carter et al., 1990; Stepp et al., 1990), suggesting that the β4 tail specifies association with the hemidesmosomal cytoskeleton.

Hemidesmosomes are complex adhesive junctions which link the basement membrane to the intracellular keratin cytoskeleton and are exclusively found in the basal cell layer of stratified and transitional epithelia (Borradori and Sonnenberg, 1996). Gene transfer studies in cultured cells have provided evidence that the unique cytoplasmic domain of β4, and specifically a region which encompasses the first pair of type-III fibronectin-like modules and the Connecting Segment, is required for association of α6β4 with the hemidesmosomal cytoskeleton (Spinardi et al., 1993). The ability of a truncated β4 subunit to exert a dominant negative effect on hemidesmosome assembly without inhibiting initial adhesion to laminin 5 (Spinardi et al., 1995) and the absence of hemidesmosomes in the skin of α6 and β4 knock-out mice (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996) suggest that α6β4 plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system. Subsequent studies have indicated that ligation of α6β4 activates an integrin-associated kinase and causes phosphorylation of the β4 cytoplasmic domain at multiple tyrosine residues (Mainiero et al., 1995). Phosphorylation of the C-terminal tyrosine residue within the Tyrosine Activation Motif (TAM) located in the Connecting Segment and of a distinct tyrosine residue in the third type II Fn-like repeat appears to be required for the recruitment of the adaptor protein Shc and the activation of Ras (Mainiero et al., 1995; 1997; Dans and Giancotti, unpublished results). In addition, there is evidence suggesting that the integrity of the TAM is required for the

association of α6β4 with the hemidesmosomal cytoskeleton and that phosphorylation of either one of its two constituent tyrosine residues abolish the incorporation of α6β4 in hemidesmosomes (Mainiero et al., 1995; Dans and Giancotti, unpublished results).

Effect of Epidermal Growth Factor on the intracellular functions of α6β4 integrin.

Prompted by the prominent role of EGF and TGF-α in controlling epithelial cell growth and migration and by the coincident expression of α6β4 and EGF-R in many epithelial tissues including the breast epithelium, we have examined the effect of EGF-R activation on the intracellular functions of α6β4. Experiments of immunoblotting with anti-phosphotyrosine (anti-P-Tyr) antibodies and immunoprecipitation followed by phosphoamino acid analysis and phosphopeptide mapping showed that activation of the EGF-R causes phosphorylation of the β4 subunit at multiple tyrosine residues, including the two elements of the TAM. Immunofluorescent analysis revealed that EGF treatment causes a deterioration of hemidesmosomes without affecting initial adhesion to laminins. Finally, Boyden chamber assays indicated that exposure to EGF results in upregulation of α6β4-mediated cell migration toward laminins. Taken together, these results indicate that EGF-dependent signals have a complex effect on α6β4 function: they cause tyrosine phosphorylation of β4, induce disassembly of hemidesmosomes, and upregulate cell migration on laminins (Mainiero et al., 1996). It is possible that the ability of activated EGF-R to coordinately disassemble hemidesmosomes and upregulate α6β4-dependent cell migration play a role during tumor progression.

The virtual identity of the phosphopeptide maps of β4 derived from vanadate and EGF treated cells suggested that the EGF-R induces phosphorylation of β4 by activating the kinase normally associated with the integrin. To identify tyrosine kinases capable of phosphorylating α6β4, we have performed immune-complex kinase assays in 293-T cells transiently transfected with constructs encoding α6 and β4 in combination with each one of various tyrosine kinases. The results indicated that the Src-family kinase Fyn can associate with α6β4 and phosphorylate the β4 subunit. In contrast, Src, Lck, and Jak-1 did not associate with α6β4, but were able to induce phosphorylation of β4. The ability of several overexpressed tyrosine kinases to phosphorylate β4 is not surprising, because the β4 tail contains mutiple potential tyrosine phosphorylation sites. Antibody-mediated cross-linking of α6β4 increased the kinase activity of the integrin-associated fraction of Fyn, without inducing further recruitment of the kinase. These observations indicate that upon overexpression Fyn can combine with α6β4 and phosphorylate the β4 tail.

The association of α6β4 with Fyn is likely to be mediated by the β4 tail because α6β1 does not interact with Fyn. The β4 tail contains three regions potentially capable of interacting with fyn: a membrane-proximal Cys-X-X-Cys motif which could interact with a similar motif in the unique N-terminal segment of fyn (a similar mechanism underlies the association of the T cell co-receptors CD4

and CD8 with Lck), a Pro-X-X-Pro sequence in the N-terminal portion of Connecting segment which could bind to the SH3 domain of Fyn, and the TAM in the C-terminal portion of Connecting Segment which may interact with the unique N-terminal segment of fyn (this portion of Fyn has been shown to interact, in a phosphorylation independent manner, with immune receptor TAMs). To gain insight to the mechanism of Fyn association with α6β4, we have used a mutational approach. The wild-type β4 subunit and several deletion mutants were introduced together with α6 and Fyn in 293-T cells. The results of coimmunoprecipitation analysis indicated that three distinct regions of the β4 tail interact with Fyn. We next wanted to identify the region of Fyn mediating the interaction with the β4 tail. Src family kinases contain at least three domains that can mediate protein-protein interactions: the unique N-terminal segment, the SH3 domain, and the SH2 domain. While the unique N-terminal segment and the SH2 domain mediate interaction with upstream signal transducers, such as receptor tyrosine kinases and immune receptors, the SH3 domain is involved in binding to substrates. The interaction of Fyn with α6β4 does not appear to be phosphorylation dependent and is therefore unlikely to be mediated by the SH2 domain. In any event, we constructed three mutant forms of Fyn: one lacking of the SH3 domain (ΔSH3), one carrying an inactivating point mutation in the SH2 domain ($SH2^-$), and a chimera consisting of the unique N-terminus (13 aa) of Fyn fused to the SH3, SH2 and kinase domain of Src (Fyn13N-Src). All mutant forms of Fyn, including Fyn13N-Src, but not Src combined with α6β4 upon transfection in 293-T cells. These results indicate that the unique N-terminus of Fyn is required and sufficient for interaction with α6β4, but do not exclude the hypothesis that other regions of Fyn also interact with β4.

Since coimmunoprecipitation experiments indicated that α6β4 is not constitutively associated with endogenous Fyn in A431 cells, HaCat keratinocytes, and 293 T cells stably transfected with β4 cDNA, we wondered if Fyn played a role in EGF-induced phosphorylation of β4 and associated with α6β4 only in response to EGF treatment. In accordance with this hypothesis, preliminary experiments indicated that a concentration of EGF, which is able to induce phosphorylation of β4, activates Fyn in a time-dependent manner. Subsequent coimmunoprecipitation experiments indicated that, upon treatment with EGF, α6β4 forms a complex with endogenous Fyn in 293-T cells stably expressing β4, A431 cells, and HaCat keratinocytes. In contrast, various β1 integrins did not form a complex with the activated EGF-R and Fyn. Immunoblotting with anti-phosphotyrosine antibodies revealed that a 180 kD mol. wt. protein formed a complex with α6β4 upon EGF stimulation. Immunoblotting with anti-EGF-R antibodies indicated that this protein corresponded to the autophosphorylated EGF-R. These results suggest that the activated EGF-R may recruit Fyn to the plasma membrane, thereby facilitating its interaction with α6β4. In accordance with this hypothesis, we have observed that increasing amounts of dominant negative (kinase-dead) Fyn suppress the phosphorylation of β4 induced by EGF. Since the phosphorylation of β4 induced by EGF has a negative effect on hemidesmosome assembly, we have tested the effect of dominant negative Fyn on hemidesmosome assembly. Interestingly, expression of dominant negative Fyn led to increased assembly of hemidesmosomes in rat 804G

cells. These results are consistent with the hypothesis that Fyn is the tyrosine kinase which downregulates the intracellular functions of α6β4 in response to EGF stimulation.

The observation that the EGF-R and its close relative Erb2/Neu are frequently overexpressed in squamous and adeno carcinomas raises the possibility that either the EGF-R or Erb2/Neu or both affect the association of α6β4 with the hemidesmosomal cytoskeleton in breast cancer cells and these events may contribute to tumor invasion. In order to examine this hypothesis, we have compared the ability of tumor cell lines expressing different levels of EGF-R or Erb2/neu to invade through matrigel in a Boyden chamber assay. These experiments revealed a correlation between increased expression of the EGF-R or Erb2/Neu and invasive ability *in vitro*. We have therefore introduced dominant negative Fyn in tumor cell lines that have significant levels of the EGF-R or Erb2/Neu and are able to assemble hemidesmosomes *in vitro*. The resulting cell lines were tested for their ability to invade through matrigel. The results indicated that expression of dominant negative Fyn suppresses in a significant way tumor cell invasion through matrigel.

CONCLUSIONS

The above described studies indicate that the expression, and possibly the function, of several integrins involved in adhesion to the basement membrane is altered during the *in vivo* progression of breast cancer in the N-Ras trangenic mouse model. The α6β4 and, to a minor extent, the α2β1 integrin are upregulated and diffusely distributed at the tumor cell surface in the primary lesions. These events are accompanied by a loss of laminin staining indicative of defective basement membrane deposition. The α3β1 integrin is diffusely distributed, but not upregulated. Since transfection of N-ras or overexpression of Erb2/Neu in breast epithelial cells does not produce the changes in integrin expression detected *in vivo*, it is likely that these changes occur as a result of tumor progression independently of a direct action of N-ras. We have thus focused on the mechanism by which tumorigenesis may lead to changes in the cell surface distribution and function of α6β4. Our results indicate that the activated EGF-R combines sequentially with Fyn and α6β4. Fyn then phosphorylates the β4 tail leading to disassembly of hemidesmosomes and increased invasive activity. Future experiments will be addressed at determining the role of the invasion mechanism we have uncovered during tumor progression *in vivo*.

REFERENCES

1. Borradori, L., and A. Sonnenberg. 1996. Hemidesmosomes: Roles in adhesion, signaling and human diseases. Curr. Opin. Cell Biol. 8:647-656.
2. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Ann. Rev. Cell. Biol. 4: 487-525.
3. Burridge, K., Petch, L.A., and L.H. Romer. 1992. Signals from focal adhesions. Current Biol. 10:537-539.
4. Carter, W.G., Kaur, P., Gil, S.G., Gahr, P.J., and E.A. Wayner. 1990. Distinct functions for integrins $\alpha 3\beta 1$ in focal adhesions and $\alpha 6\beta 4$/Bullous Pemphigoid Antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. J. Cell Biol. 111:3141-3154.
5. Chen, W.T., Hasegawa, E., Hasegawa, T., Weinstock, C., and K.M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. J. Cell Biol. 100: 1103-1114.
6. Chen, W.-T., Wang, J., Hasegawa, T., Yamada, S.S., and K.M. Yamada. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J. Cell Biol. 103:1649-1661.
7. Dairkee, S.H., Blayney, C., Smith, H.S., and A.J. Ackett. 1985. Monoclonal antibody that defines human myoepithelium. Proc. Natl. Acad. Sci. U.S.A. 82:7409-7413.
8. Damsky, C.H., Knudsen, K.A., Bradley, D., Buck, C.A., and A.F. Horowitz. 1985. Distribution of the cell substratum attachement (CSAT) antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. 100:1528-1539.
9. Dowling, J., Yu, Q.-C., and E. Fuchs. 1996. $\beta 4$ integrin is required for hemidesmosome formation, cell adhesion and cell survival. J. Cell Biol. 134:559-572.
10. Dulbecco, R., Armstrong, B., and R. Allen. 1988. Reversion toward an earlier stage of differentiation and loss of polarity during progression of N-methyl-N-nitrosourea induced rat mammary tumors. Proc. Natl. Acad. Sci. U.S.A. 85:9292-9296.
11. Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A., and M. Le Meur. 1996. Absence of integrin $\alpha 6$ leads to epidermolysis bullosa and neonatal death in mice. Nature Genet. 13:370-373.
12. F.G. Giancotti. 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. 9:691-700.
13. Giancotti, F.G., Comoglio, P.M., and G. Tarone. 1986. A 135,000 molecular weight plasma membrane glycoprotein involved in fibronectin-mediated cell adhesion. Immunofluorescence localization in normal and RSV-transformed fibroblasts. Exp.
14. Giancotti, F.G., and F. Mainiero. 1994. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim. Biophys. Acta. 1198:47-64.
15. Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell 60:849-859.

16. Hayman, E.G., Engvall, E., and E. Ruoslahti. 1981. Concomitant loss of fibronectin and laminin from transformed rat kidney cells. J. Cell Biol. 88:352-357.
17. Hirst, R., Horwitz, A.F., Buck, C.A., and L. Rohrschneider. 1986. Phosphorylation of the fibronectrin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. Proc. Natl. Acad. Sci. U.S.A. 83:6470-6474.
18. Hogervorst, F., Kuikman, I., von dem Borne, A.E.G., and A. Sonnenberg. 1990. Cloning and sequence analysis of β4 cDNA: An integrin subunit that contains a unique 118 kd cytoplasmic domain. E.M.B.O. J. 9:745-770.
19. R.O. Hynes. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11-25.
20. Juliano, R.L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. J. Cell Biol. 120:577-585.
21. Koukoulis, G.K., Virtanen, E., Korhonen, M., Laitinen, L., Quaranta, V., and V.E. Gould. 1991. Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Am. J. Pathol. 139:787-799.
22. Maniero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumemberg, M., Westwick, J.K., Der, C.J., and F.G. Giancotti. 1997. The coupling of α6β4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. E.M.B.O. J. 9:2365-2375.
23. Mainiero, F., Pepe, A., Spinardi, L., Wary, K.K., Ammad, M., Schlessinger, J., and F.G. Giancotti. 1995. Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. E.M.B.O. J. 14: 4470-4481.
24. Mainiero, F., Pepe, A., Yeon, M., Ren, Y-L., and F.G. Giancotti. 1996. The intracellular functions of $\alpha_6\beta_4$ integrin are regulated by EGF. J. Cell Biol. 134: 241-253.
25. Mangues, R., Seidman, E., Gordon, G.W., and A. Pellicer. 1992. Over-expression of the N-*ras* protooncogene, not somatic mutational activation associated with malignant tumors in transgenic mice. Oncogene 7:2073-2076.
26. Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and P. Leder. 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activayed c-*neu* oncogene. Cell 54:105-115.
27. Natali, P.G., Nicotra, M.R., Botti, C., Mottolese, M., Bigotti, A., and O. Segatto. 1992. Changes in expression of $\alpha_6\beta_4$ integrin heterodimer in primary and metastatic brest cancer. Br. J. Cancer 66:318-322.
28. Petersen, O.W., Ronnov-Jessen, L., Howlett, A.R., and M.J. Bissell. 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 89:9064-9068.
29. Plantefaber, L.C., and R.O. Hynes. 1989. Changes in integrin receptors on oncogenically transformed cells. Cell 56:281-290.
30. P.S. Rudland. 1987. Stem cells and development of mammary cancers in experimental rats and in humans. Cancer Metastasis Rev. 6:55-83.

31. E. Ruoslahti. 1984. Fibronectin in cell adhesion and invasion. Cancer Metastasis Rev. 3:34-51.
32. Ruoslahti, E., and F.G. Giancotti. 1989. Integrins in tumor dissemination. Cancer Cells 1:119-126.
33. Schreiner, C.L., Fisher, M., Hussein, S., and R.L. Juliano. 1991. Increased tumorigenicity of fibronectin receptor deficient Chinese hamster ovary cell variants. Cancer Res. 51:1738-1740.
34. Sinn, E., Muller, W.J., Pattengale, P.K., Tepler, I., Wallace, R., and P. Leder. 1987. Coexpression of MMTV/v-H-*ras* and MMTV/c-*myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. Cell 49:465-475.
35. Spinardi, L., Einheber, S., Cullen., T., Milner, T.A., and F.G. Giancotti. 1995. A recombinant tail-less integrin β4 subunit disrupts hemidesmosomes, but does not suppress α6β4-mediated cell adhesion to laminins. J. Cell Biol. 129:473-487.
36. Spinardi, L., Ren, Y.-L., Sanders, R., and F.G. Giancotti. 1993.The β4 subunit cytoplasmic domain mediates the interaction of α6β4 integrin with the cytoskeleton of hemidesmosomes. Mol. Biol. Cell. 4:871-884.
37. Stepp, M.A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and I.K. Gipson. 1990. Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. Proc. Natl. Acad. Sci. USA. 87:8970-8974.
38. Streuli, C.H., Bailey, N., and M.J. Bissell. 1991. Control of mammary epithelial differentiation: basement membrane induced tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. J. Cell Biol. 115:1383-1395.
39. Suzuki, S., and Y.Naitoh. 1990. Amino acid sequence of a novel integrin β4 subunit and primary expression of the mRNA in epithelial cells. E.M.B.O. J. 9:757-763.
40. B.E. Symington. 1990. Fibronectin receptor overexpression and loss of transformed phenotype in a stable variant of the K562 cell line. Cell Regul. 1:637-648.
41. Tapley, P., Horwitz, A.F., Buck, C.A., Duggan, K., and L. Rohrschneider. 1989. Integrins isolated from RSV-transformed chicken embryo fibroblasts. Oncogene 4:325-333.
42. Trask, D.K., Band, V., Zajchowski, D.A., Yawsen, P., Suh, T., and R. Sager. 1990. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 87:2319-2323.
43. van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M., and A. Sonnenberg. 1996. Epithelial detachment due to absence of hemidesmosomes in integrin β4 null mice. Nature Genet. 13:366-369.
44. Wary, K.K., Mainiero, F., Isakoff, S., Marcantonio, E.E., and F.G. Giancotti. 1996. The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell 87: 733-743.
45. Wellings, S.R., Jensen, H.M., and R.G. Marcum. 1975. An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. J. Natl. Cancer Inst. 55:231-273.
46. Zutter, M.M., Mazoujian, G., and S.A. Santoro. 1990. Decreased expression of integrin adhesive protein receptors in adhenocarcinoma of the breast. Am. J.

Pathol. 137:863-870.

PUBLICATIONS

1. Mainiero, F., Pepe, A., Spinardi, L., Wary, K., Ammad, M., Schlessinger, J., and F.G. Giancotti. 1995. Signal transduction by the α6β4 integrin: distinct β4 sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. E.M.B.O. J. 14:4470-4481.

2. Giancotti, F.G. 1996. Signal transduction by the α6β4 integrin: charting the path between laminin binding and nuclear events. J. Cell Science 109:1165-1172 [Review Article].

3. Mainiero, F., Pepe, A., Yeon, M., Ren, Y.-L., and F.G. Giancotti. 1996. The intracellular functions of α6β4 integrin are regulated by EGF. J. Cell Biol. 134:241-253.

4. Klein, S., Bikfalvi, A., Birkenmeier, T.M., Giancotti, F.G., and D.B. Rifkin. 1996. Integrin regulation by endogenous expression of 18 kD Fibroblast Growth Factor-2. J. Biol. Chem. 271:22583-22590.

5. Jones, J., Sujiyama, M., Giancotti, F.G., Speight, P.M., and F.M. Watt. 1996. Transfection of β4 integrin subunit into a neoplastic keratinocyte line fails to restore terminal differentiation capacity or influence proliferation. Cell Adhesion and Communication 4:307-316.

6. Wary, K.K., Mainiero, F., Isakoff, S., Marcantonio, E.E., and F.G. Giancotti. 1996 The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell 87:733-743.

7. Mainiero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumemberg, M., Westwick, J.K., Der, C., and F.G. Giancotti. 1997. The coupling of α6β4 integrin to the Ras-MAP Kinase pathways mediated by Shc controls keratinocyte proliferation. E.M.B.O. J. 16:2365-2375.

8. Giancotti, F.G. 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Op. Cell Biol. 9:691-700 [Review Article].

9. Sanders, R.J., Mainiero, F., and F.G. Giancotti. 1998. The role of integrins in tumorigenesis and metastasis. Cancer Invest. 16:329-344 [Review Article].

10. Murgia, C., Blaikie, P., Dans, M., Kim, N., Petrie, H., and F.G. Giancotti. 1998 Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin β4 subunit cytoplasmic domain. E.M.B.O. J. *17*:3940-3951.

11. Pozzi, A., Wary, K.K., Giancotti, F.G., and H.A. Gardner. 1998. Integrin α1β1 mediates a unique collagen dependent proliferation pathway in vivo. J. Cell Biol. 142:587-594.

12. Munger, J.S., Harpel, J.G., Giancotti, F.G., and D.B. Rifkin. 1998. Interactions between growth factors and integrins: latent forms of Transforming Growth Factor-β are ligands for the integrin αvβ1. Mol. Biol. Cell 9:2627-2638.

13. Wary, K.K., Mariotti, A., Zurzolo, C., and F.G. Giancotti. 1998. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 94:625-634.

14. Lora, J.M., Rowader, K.E., Soares, L., Giancotti, F.G., and K.S. Zaret. 1998. α3β1 integrin as a critical mediator of the hepatic differentiation response to the extracellular matrix. Hepatology 28:1095-1104.

LIST OF PERSONNEL

Anna Maria Curatola, Ph.D., Principal Investigator.
Filippo G. Giancotti, M.D., Ph.D., Co-principal Investigator.
Agnese Mariotti, Ph.D., Postdoctoral Associate.
Julie Bian, B.A., Technician.

The EMBO Journal vol.14 no.18 pp.4470–4481, 1995

Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes

Fabrizio Mainiero, Angela Pepe, Kishore K.Wary, Laura Spinardi[1], Moosa Mohammadi[2], Joseph Schlessinger[2] and Filippo G.Giancotti[3]

Departments of Pathology and [2]Pharmacology, Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, NY 10016, USA
[1]Present address: DIBIT, Istituto Scientifico S. Raffaele, Milan, 20132, Italy

[3]Corresponding author

We have examined the mechanism of signal transduction by the hemidesmosomal integrin $\alpha_6\beta_4$, a laminin receptor involved in morphogenesis and tumor progression. Immunoprecipitation and immune complex kinase assays indicated that antibody- or laminin-induced ligation of $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit in intact cells and that this event is mediated by a protein kinase(s) physically associated with the integrin. Co-immunoprecipitation and GST fusion protein binding experiments showed that the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β_4 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor Grb2, thereby potentially linking $\alpha_6\beta_4$ to the *ras* pathway. The β_4 subunit was found to be phosphorylated at multiple tyrosine residues *in vivo*, including a tyrosine-based activation motif (TAM) resembling those found in T and B cell receptors. Phenylalanine substitutions at the β_4 TAM disrupted association of $\alpha_6\beta_4$ with hemidesmosomes, but did not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2. These results indicate that signal transduction by the $\alpha_6\beta_4$ integrin is mediated by an associated tyrosine kinase and that phosphorylation of distinct sites in the β_4 tail mediates assembly of the hemidesmosomal cytoskeleton and recruitment of Shc/Grb2.

Keywords: hemidesmosomes/integrins/Shc/signaling/tyrosine phosphorylation

Introduction

Basement membranes provide cells with positional cues which can affect their proliferation and differentiation (Adams and Watt, 1993). It is now clear that cell–matrix interactions are in large part mediated by integrins (Ruoslahti, 1991; Hynes, 1992) and that ligation of integrins results in intracellular signaling (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994). Many of the influences of basement membranes on cellular behavior can be recapitulated *in vitro* by laminins or blocked with anti-laminin antibodies (Adams and Watt, 1993). It is therefore important to elucidate the mechanisms by which binding of laminins to integrins results in the activation of signal transduction pathways.

Laminins are a growing family of obligatory components of basement membranes expressed in a tissue- and development-specific manner (Engvall, 1993). At least six cell surface receptors, including various β_1 integrins and the $\alpha_6\beta_4$ integrin, have been implicated in binding to laminins and in many cases their binding specificities appear to overlap (Mercurio, 1990; Hynes, 1992). Cell adhesion to laminins, however, results in different patterns of gene expression depending on cell type and perhaps developmental stage (Di Persio *et al.*, 1991; Roskelley *et al.*, 1994), suggesting that specific signals may result from the engagement of distinct laminin binding integrins in different cells.

Focal adhesion kinase (FAK) (Shaller *et al.*, 1992) has been implicated in signaling from β_1 and β_3 integrins (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Lipfert *et al.*, 1992). There is evidence suggesting that FAK can link integrins to the *ras* signaling pathway (Schlaepfer *et al.*, 1994), as well as induce intracellular changes which are potentially important for assembly of the actin cytoskeleton, such as phosphorylation of paxillin and tensin (Burridge *et al.*, 1992; Bockholt and Burridge, 1993) and activation of *Rho* (McNamee *et al.*, 1992; Chong *et al.*, 1994). However, the mechanisms by which β_1 and β_3 integrins activate FAK have remained elusive so far. In particular, since ligation of the platelet integrin $\alpha_{IIb}\beta_3$ causes a cascade of tyrosine phosphorylation events prior to activation of FAK (Huang *et al.*, 1993) and since the latter event requires an additional co-stimulus provided by an agonist receptor (Shattil *et al.*, 1994), it is possible that FAK does not lie immediately downstream of the integrins. Thus although these results establish the role of integrins in signaling, they do not clarify how laminin-derived signals are transduced at the plasma membrane and how specificity of signaling is achieved.

The $\alpha_6\beta_4$ integrin is a receptor for various laminins and binds with the highest relative affinity to laminins 4 and 5 (Spinardi *et al.*, 1995). The highest levels of expression of $\alpha_6\beta_4$ are observed in the basal cell layer of stratified epithelia (Kajiji *et al.*, 1989), at the ends of endothelial sprouts during angiogenesis (Enenstein and Kramer, 1994), in Schwann cells at the onset of myelination (Einheber *et al.*, 1993) and in $CD4^-$ $CD8^-$ pre-T lymphocytes entering the thymus (Wadsworth *et al.*, 1992), suggesting the involvement of $\alpha_6\beta_4$ in various morphogenetic events. In addition, increased levels of $\alpha_6\beta_4$ are expressed in squamous, but not basal, carcinomas in humans (Kimmel and Carey, 1986; Savoia *et al.*, 1993) and suprabasal expression of $\alpha_6\beta_4$ is associated with malignant progression during mouse skin carcinogenesis (Tennenbaum *et al.*, 1993). Elucidation of the signal transduction mechanism of the $\alpha_6\beta_4$ integrin may, therefore, help us to understand

the differential effects induced by basement membranes in different normal cell types, as well as the significance of $\alpha_6\beta_4$ up-regulation in cancer cells.

The cytoplasmic domain of β_4 may provide the $\alpha_6\beta_4$ integrin with unique cytoskeletal and signaling interactions. The β_4 tail is very large (~1000 amino acids) and bears no homology with the short cytoplasmic domains of other known β subunits, including the β_1 and β_3 integrins, which are known to activate FAK. It contains, toward its C-terminus, two pairs of type III fibronectin (Fn)-like modules interrupted by a 142 amino acid long sequence (Connecting Segment) (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Furthermore, in contrast to the β_1 and β_3 integrins, which localize to focal adhesions, the $\alpha_6\beta_4$ integrin is found concentrated in hemidesmosomes (Carter *et al.*, 1990; Stepp *et al.*, 1990). Recent results demonstrate that $\alpha_6\beta_4$ plays a necessary role in the assembly of hemidesmosomes (Spinardi *et al.*, 1995). Upon binding to extracellular ligand, $\alpha_6\beta_4$ associates with cytoskeletal elements of hemidesmosomes, thereby linking the basement membrane to the keratin filament system. This $\alpha_6\beta_4$ function requires a specific region of the unique β_4 cytoplasmic domain, comprising the first pair of type III Fn-like repeats and the Connecting Segment (Spinardi *et al.*, 1993). Collectively, the unique structure, subcellular localization and cytoskeletal interactions of $\alpha_6\beta_4$ suggest that it may transduce intracellular signals by mechanisms distinct from those used by other integrins.

We here provide evidence that signal transduction by the $\alpha_6\beta_4$ integrin is mediated by an associated tyrosine kinase capable of phosphorylating the β_4 subunit. Mutations at a tyrosine activation motif (TAM) in the β_4 tail prevented the incorporation of $\alpha_6\beta_4$ into hemidesmosomes, but not the binding of Shc and Grb2, indicating that these two functions are mediated by phosphorylation of distinct integrin motifs.

Results

Ligation of the $\alpha_6\beta_4$ integrin induces tyrosine phosphorylation of the β_4 subunit

To examine the role of tyrosine phosphorylation in signal transduction by the $\alpha_6\beta_4$ integrin we asked if ligation of the extracellular portion of the integrin resulted in tyrosine phosphorylation of its component α or β subunits. To obtain selective ligation of $\alpha_6\beta_4$ in the absence of any concomitant stimulation caused by growth factors or cell shape changes, human epidermoid carcinoma A431 cells were serum starved, detached and then incubated in suspension with polystyrene beads coated with the anti-β_4 monoclonal antibody 3E1 or the control anti-MHC monoclonal antibody W6.32. Tyrosine phosphorylation of $\alpha_6\beta_4$ was monitored over time by immunoprecipitation with the 3E1 antibody followed by immunoblotting with anti-phosphotyrosine (P-Tyr) antibodies. As shown in Figure 1, the β_4 subunit was transiently phosphorylated on tyrosine in cells treated with anti-β_4 beads, but was not significantly phosphorylated in cells treated with control beads. In addition, no tyrosine phosphorylation of β_4 was observed in cells incubated with soluble 3E1 antibodies (not shown). These observations indicate that antibody-mediated cross-linking of $\alpha_6\beta_4$ results in activation of a tyrosine kinase capable of phosphorylating the β_4 subunit.

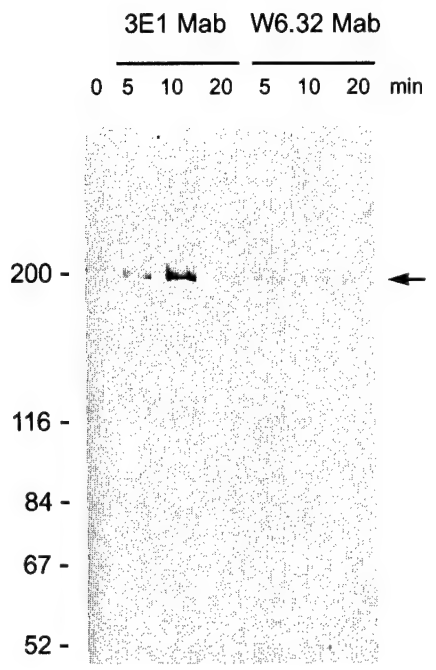


Fig. 1. Ligation of the extracellular portion of $\alpha_6\beta_4$ induces tyrosine phosphorylation of β_4. Serum-starved A431 cells were stimulated in suspension for the indicated times with beads coated either with the anti-β_4 monoclonal antibody 3E1 or the anti-MHC monoclonal antibody W6.32. Equal amounts of total proteins were immunoprecipitated with the 3E1 antibody and probed by immunoblotting with polyclonal anti-P-Tyr antibodies. Molecular weight markers are indicated in kDa.

To test whether the $\alpha_6\beta_4$ integrin is associated with cytoplasmic protein kinase(s), immune complex kinase assays were performed. The A431 cells, which express several β_1 integrins, as well as $\alpha_6\beta_4$, were immunoprecipitated with the monoclonal antibodies 3E1 and AIIB2, directed against the β_4 and the β_1 integrin subunits respectively. The immunoprecipitated samples were subjected to kinase assay and analyzed by SDS–PAGE. As shown in Figure 2A, incubation of the anti-β_4 immunoprecipitate with [γ-^{32}P]ATP resulted in significant phosphorylation of a 200 kDa protein corresponding to β_4, as well as lower level phosphorylation of an additional 140 kDa protein. Occasionally, additional proteins with apparent molecular masses of 50–70 kDa also underwent specific phosphorylation in the *in vitro* reaction. In contrast, despite the presence of a tyrosine phosphorylation consensus site in the cytoplasmic domain of the β_1 subunit (Tamkun *et al.*, 1986), incubation of the anti-β_1 immunoprecipitate with [γ-^{32}P]ATP did not yield any specific phosphorylation product under these experimental conditions. Similar results were obtained with Lovo human colon carcinoma and 804G rat bladder carcinoma cells, which both express endogenous $\alpha_6\beta_4$. In addition, analysis of 804G cells expressing either a wild-type or a tail-less human β_4 subunit from cDNA indicated that while the full-length subunit was efficiently phosphorylated in the immune complex kinase assay, the truncated protein was not (Figure 2A). These results indicate that the β_4 subunit is phosphorylated *in vitro* by a protein kinase(s) associated

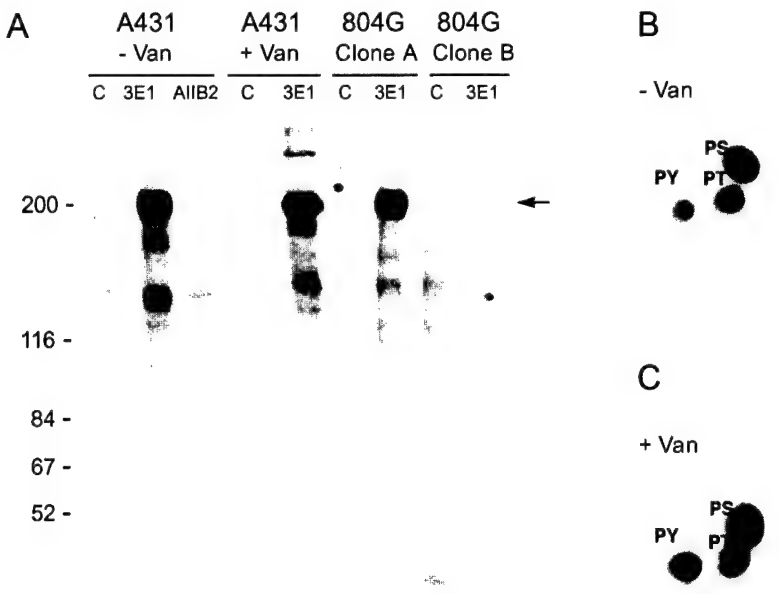


Fig. 2. The $\alpha_6\beta_4$ integrin is associated with a protein kinase(s) which can phosphorylate the β_4 subunit *in vitro*. (**A**) Phosphorylation of β_4 in an immune complex kinase assay. A431 cells were either directly extracted with Brij 96 buffer or treated with 10 μM vanadate for 10 min prior to extraction. Rat 804G cells expressing a recombinant wild-type (clone A) or tail-less human β_4 subunit (clone B) were directly lysed with Brij 96 buffer. Equal amounts of total proteins were immunoprecipitated with control rabbit anti-mouse IgGs (C), anti-β_4 monoclonal antibody 3E1 (3E1) or anti-β_1 monoclonal antibody AIIB2 (AIIB2). The samples were subjected to an *in vitro* kinase assay and separated by SDS–PAGE. The arrow points to β_4. (**B**) Phosphoamino acid analysis of *in vitro* labeled β_4 from untreated A431 cells (–Van). (**C**) Phosphoamino acid analysis of *in vitro* labeled β_4 from vanadate-treated A431 cells (+Van). Identical amounts of radioactivity were loaded in (B) and (C).

with the integrin and that this event requires an intact β_4 cytoplasmic domain.

Phosphoamino acid analysis indicated that the *in vitro* phosphorylated β_4 subunit contained a significant amount of phosphotyrosine, in addition to phosphothreonine and phosphoserine (Figure 2B). The incorporation of phosphate on tyrosine, threonine and serine residues was reduced, but not suppressed, if the immunoprecipitate was washed repeatedly under stringent conditions prior to the reaction (see Materials and methods), suggesting that the phosphorylation of β_4 was specific and was not caused by kinases contaminating the immunoprecipitate. Since the intracellular portion of $\alpha_6\beta_4$ does not contain a protein kinase domain, these results suggest that the integrin is physically associated with protein kinase(s) capable of phosphorylating β_4 on tyrosine, threonine and serine residues *in vitro*. Although it is likely that $\alpha_6\beta_4$ is associated with two protein kinases with distinct amino acid selectivity, these results do not exclude the possibility of an association with a dual specificity kinase.

Pretreatment of the cells with 10 μM vanadate prior to detergent extraction and immune complex kinase assay enhanced the amount of phosphotyrosine recovered from β_4 after the reaction (compare Figure 2B and C), suggesting that tyrosine phosphorylation of β_4 is subject to negative regulation by protein tyrosine phosphatases. The relatively rapid time course of β_4 dephosphorylation observed after antibody-mediated engagement of the integrin (Figure 1) and the ability of micromolar concentrations of sodium orthovanadate to induce significant tyrosine phosphorylation of β_4 in intact cells (see Figure 7A and B, below) are also consistent with this hypothesis. Ligation of $\alpha_6\beta_4$ with anti-β_4-coated beads prior to extraction and immune complex kinase assay only led to a modest increase in the amount of phosphotyrosine recovered from β_4 after the reaction, suggesting that the association of $\alpha_6\beta_4$ with a tyrosine kinase is constitutive (data not shown). These results suggest that the antibodies to $\alpha_6\beta_4$ elicit tyrosine phosphorylation of the β_4 subunit *in vivo* by inducing dimerization or oligomerization of the integrin on the plasma membrane and thereby activating an associated protein tyrosine kinase and/or bringing it into close proximity to its target sequences in β_4.

Association of $\alpha_6\beta_4$ with Shc and Grb2

Since tyrosine phosphorylation regulates the recruitment of SH2 domain molecules to activated cell surface receptors, we examined the possible involvement of SH2 domain proteins in signaling by $\alpha_6\beta_4$. To test if the adaptor protein Shc formed a complex with tyrosine-phosphorylated $\alpha_6\beta_4$, A431 cells were stimulated with anti-β_4 or anti-MHC beads and the resulting extracts were either immunoprecipitated with anti-β_4 antibodies and probed by immunoblotting with anti-Shc antibodies or immunoprecipitated with anti-Shc antibodies and probed with anti-β_4 antibodies. The results showed that $p52^{Shc}$ is co-immunoprecipitated with $\alpha_6\beta_4$ from cells incubated with anti-β_4 beads, but not from those treated with anti-MHC beads (Figure 3A). Although the other two Shc isoforms, $p46^{Shc}$ and $p66^{Shc}$, are expressed at levels comparable with that of $p52^{Shc}$ in A431 cells (Pelicci, 1992) and are recognized by the antibodies used in this study, only a very modest amount of $p46^{Shc}$ and no $p66^{Shc}$ was detected in association with $\alpha_6\beta_4$. In addition, in accordance with the observation that $\alpha_6\beta_4$ does not contain tyrosine phosphorylation sites conforming to the consensus for binding to the p85 subunit of phosphatidylinositol-3-hydroxyl kinase or phospholipase C-γ (Songyang *et al.*, 1993), we did not detect an association of these SH2 molecules with tyrosine-phosphorylated $\alpha_6\beta_4$. Taken

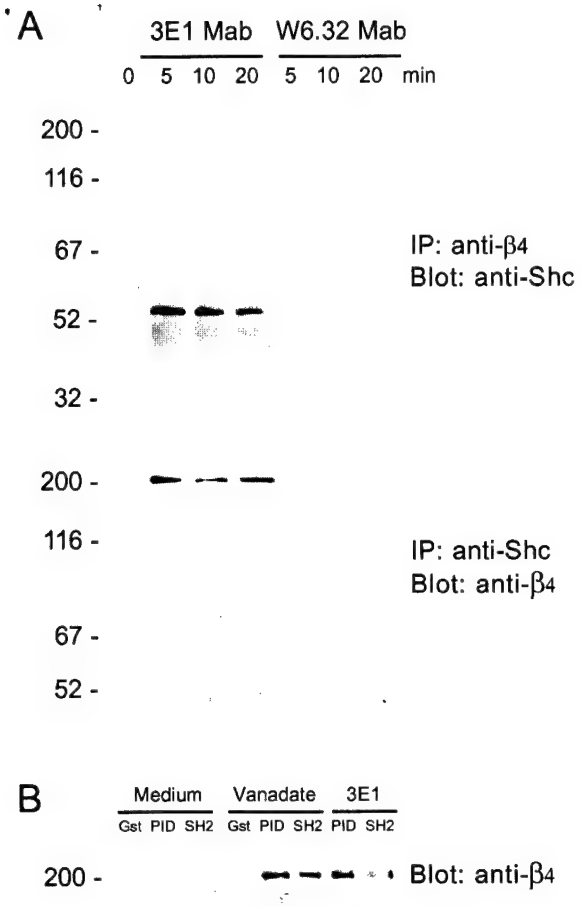


Fig. 3. Association of $\alpha_6\beta_4$ with Shc and Grb2. (**A**) Shc forms a complex with activated $\alpha_6\beta_4$ in intact cells. A431 cells were stimulated as indicated in Figure 1. Equal amounts of total proteins were immunoprecipitated with rabbit anti-β_4 peptide serum (top) or rabbit anti-Shc serum (bottom). The samples were probed by immunoblotting with anti-Shc monoclonal antibody (top) or anti-β_4 monoclonal antibody 450-11A (bottom). (**B**) Binding of the Shc PID and SH2 domains to β_4. Rat 804G cells expressing the human wild-type β_4 subunit (clone A) were serum starved and treated with medium alone, 100 μM sodium orthovanadate plus 3 mM H_2O_2 or stimulated in suspension with anti-β_4 beads for 10 min at 37°C. Denatured lysates were incubated with glutathione–agarose beads carrying the GST leader protein alone (GST) or GST–Shc PID domain (PID) or GST–Shc SH2 domain (SH2). Bound proteins were separated by SDS–PAGE and analyzed by immunoblotting with polyclonal anti-β_4 antibodies.

together, these results indicate that $p52^{Shc}$ forms a specific complex with the activated $\alpha_6\beta_4$ integrin.

We next wondered if Shc could interact directly with the tyrosine-phosphorylated β_4 subunit and whether the interaction was mediated by the SH2 domain or the Phosphotyrosine Interaction Domain (PID) of Shc. 804G cells expressing a recombinant wild-type β_4 subunit were treated with sodium orthovanadate or incubated with anti-β_4 beads to induce β_4 phosphorylation. The extracts were denatured by heating in 1% SDS and incubated with agarose-immobilized GST fusion proteins encoding either the PID or the SH2 domain of Shc. Bound proteins were analyzed by immunoblotting with anti-β_4 antibodies. As shown in Figure 3B, both the PID and the SH2 domain of Shc bound to the β_4 subunit extracted from cells treated with vanadate or anti-β_4 antibodies, but not to β_4 from control, untreated cells. These results suggest that the

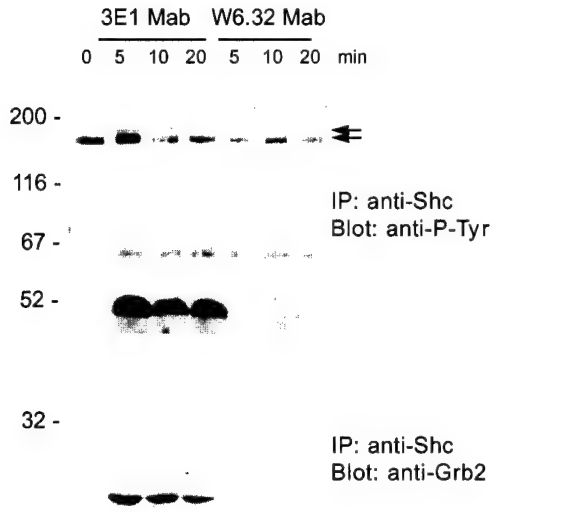


Fig. 4. Recruitment of Shc and Grb2 to the activated $\alpha_6\beta_4$ integrin. A431 cells were stimulated as indicated in Figure 1. Equal amounts of total proteins were immunoprecipitated with rabbit anti-Shc serum. The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20 (top panel) or with monoclonal anti-Grb2 antibody (bottom panel).

tyrosine-phosphorylated β_4 subunit can interact directly with both the PID and the SH2 domain of Shc.

To examine the effect of $\alpha_6\beta_4$ ligation on tyrosine phosphorylation of Shc, A431 cells were incubated with anti-β_4 or control beads and immunoprecipitated with anti-Shc antibodies. The samples were analyzed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 4 (upper panel), treatment of the cells with anti-β_4, but not control, beads led to tyrosine phosphorylation of $p52^{Shc}$. Two tyrosine-phosphorylated proteins were co-immunoprecipitated with Shc, a 195 kDa component which appeared to be constitutively associated with Shc and was not investigated further (lower arrow) and a 200 kDa molecule which was detected in association with Shc in cells treated with anti-β_4, but not control, antibodies (upper arrow). Reprobing of the blot with the anti-β_4 monoclonal antibody 450-11A revealed that this latter protein corresponded to the tyrosine-phosphorylated β_4 subunit. These results indicate that upon forming a complex with activated $\alpha_6\beta_4$, $p52^{Shc}$ becomes phosphorylated on tyrosine.

To examine the possibility that tyrosine-phosphorylated Shc associates with Grb2 upon ligation of $\alpha_6\beta_4$, extracts derived from A431 cells treated with anti-β_4 or control beads were subjected to immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-Grb2 antibodies. The result indicated that Grb2 forms a complex with Shc in cells stimulated with anti-β_4, but not control, beads (Figure 4, lower panel). Grb2 could also be detected in anti-β_4 immunoprecipitates from stimulated cells, but in lower amounts than in the anti-Shc immunoprecipitates. Together with the observation that the β_4 tail does not contain consensus Grb2 binding motifs (Songyang *et al.*, 1993), these results suggest that the association of Grb2 with $\alpha_6\beta_4$ is mediated by $p52^{Shc}$ and contingent upon its tyrosine phosphorylation. Collectively these findings indicate that the two adaptors Shc and Grb2 interact sequentially with $\alpha_6\beta_4$, thereby potentially linking the integrin to the *ras* signaling pathway.

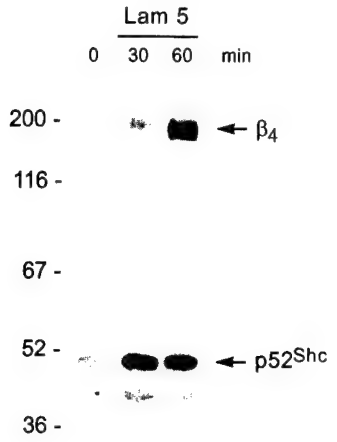


Fig. 5. Adhesion to laminin 5 results in tyrosine phosphorylation of the β_4 subunit and Shc. A431 cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes for the indicated times. After extraction, the samples were immunoprecipitated with polyclonal antibodies to β_4 (top panel) or Shc (bottom panel). The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20.

Cell adhesion to laminin 5 results in tyrosine phosphorylation of β_4 and p52^{Shc}

We next asked whether the above-described intracellular events also occurred in response to engagement of $\alpha_6\beta_4$ by extracellular matrix ligand. A431 cells were serum starved, detached and either kept in suspension or plated for different times on laminin 5 matrix-coated plates. Tyrosine phosphorylation of β_4 and p52^{Shc} was monitored by immunoprecipitation with specific antibodies followed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 5, cell adhesion to laminin 5 resulted in tyrosine phosphorylation of β_4, p52^{Shc} and, to a minor extent, p46^{Shc}, but these events occurred with slower kinetics than in cells incubated in suspension with anti-β_4 beads. Presumably this is because ligation of integrins during cell adhesion to extracellular matrix ligand does not occur as rapidly and synchronously as during incubation with antibody-coated beads. These results suggest that the binding of extracellular matrix ligands to $\alpha_6\beta_4$ results in the same intracellular changes that are observed upon antibody-mediated ligation of the integrin.

Phosphorylation of a tyrosine-based activation motif (TAM) in the β_4 cytoplasmic domain

To assess the biological significance of β_4 phosphorylation, we sought to examine the tyrosine phosphorylation sites in β_4. Preliminary studies using a combination of deletion mutagenesis and immunoblotting with anti-P-Tyr antibodies pointed to the presence of major tyrosine phosphorylation sites in the β_4 Connecting Segment (data not shown). Inspection of the amino acid sequence of the Connecting Segment revealed three potential tyrosine phosphorylation sites: Tyr1343, Tyr1422 and Tyr1440. We noted that the closely spaced Tyr1422 and Tyr1440 are embedded in very similar amino acid contexts. In particular, both residues are followed at position +3 by a leucine. Tandem tyrosine phosphorylation sites with a leucine at position +3 play a critical role in signal transduction by antigen receptors and are commonly referred to as TAMs

β4 Integrin Subunit	R D	Y 1422	N S	L	T R S E H S H S T T L P R D	Y 1440	S T	L
h ζ 1	Q L	Y	N E	L	N L G R R E E - - - - - - -	Y	D V	L
h ζ 2	G L	Y	N E	L	Q K D K M A E A - - - - - -	Y	S E	I
h ζ 3	G L	Y	Q G	L	S T A T K D T - - - - - - -	Y	D A	L
h CD3 γ	Q L	Y	Q P	L	K D R E D D Q - - - - - - -	Y	S H	L
h CD3 ε	P D	Y	E P	I	R K G Q R D L - - - - - - -	Y	S G	L
h CD3 δ	Q V	Y	Q P	L	R D R D D A Q - - - - - - -	Y	S H	L
h FcγR IIA	G G	Y	M T	L	N P R A P T D D D K N I - -	Y	L T	L
r FcεRγ	A V	Y	T G	L	N T R N Q E T - - - - - - -	Y	E T	L
r FcεRβ	R L	Y	E E	L	H V Y S P I - - - - - - - -	Y	S A	L
m Igα	N L	Y	E G	L	N L D D C S M - - - - - - -	Y	E D	I
m Igβ	H T	Y	E G	L	N I D Q T A T - - - - - - -	Y	E D	I
BLV gp30	S D	Y	Q A	L	L P S A P E I - - - - - - -	Y	S H	L

Fig. 6. Alignment of tyrosine activation motifs (TAMs) within the cytoplasmic domains of the human integrin β_4 subunit, the human TCR ζ and CD3 chains, human FcγRIIA, the rat FcεRβ and γ chains, the mouse Igα and β chains associated with IgM and IgD on B cells and the envelope glycoprotein (gp30) of bovine leukemia virus (BLV).

or antigen recognition activation motifs (ARAMs) (Weiss and Littman, 1994). Figure 6 shows an alignment of the β_4 TAM with the other previously identified TAMs, which include those present in the T cell receptor (TCR), B cell receptor (BCR), Fcε and Fcγ receptors and the bovine leukemia virus gp30 glycoprotein.

To determine if the β_4 TAM sequence is phosphorylated *in vivo* and examine the physiological significance of this event, we generated and then introduced into 804G cells β_4 cDNAs carrying either individual phenylalanine substitutions at Tyr1343, Tyr1422 and Tyr1440 or a combined replacement of Tyr1422 and Tyr1440. Fluorescence activated cell sorting (FACS) analysis indicated that the cDNA encoded mutant subunits Y1343F, Y1422F, Y1440F and Y1422F/Y1440F were expressed at the cell surface at levels comparable with that of wild-type recombinant β_4.

Wild-type β_4 and phenylalanine mutant subunits were examined by *in vivo* labeling and phosphopeptide mapping. Since antibody- or ligand-induced cross-linking of $\alpha_6\beta_4$ did not produce the high level tyrosine phosphorylation of β_4 required for mapping, tyrosine phosphorylation of β_4 was obtained by exposing the cells to vanadate. Preliminary experiments of [^{32}P]orthophosphate labeling and phosphoamino acid analysis revealed that the wild-type β_4 subunit is constitutively phosphorylated on serine residues *in vivo*, but becomes phosphorylated on tyrosine residues upon vanadate treatment (Figure 7A and B). *Staphylococcus* V8 protease digestion of wild-type β_4 from vanadate-treated cells yielded five major phosphopeptides (S1–S3, Y5 and Y6) and a number of minor phosphopeptides (Y1–Y4) (Figure 7, top panel). Phosphoamino acid analysis of individual phosphopeptides indicated that the major phosphopeptides S1–S3 contain exclusively radioactive phosphoserine. This observation is consistent with their presence in phosphopeptide maps of β_4 isolated from unstimulated cells. In contrast, the two major phosphopeptides Y5 and Y6, as well as the minor phosphopeptides Y1–Y4, which were only detected in stimulated cells, were found to contain exclusively phosphotyrosine. We concluded that β_4 is phosphorylated at multiple tyrosine residues *in vivo*.

We next examined the phosphopeptide maps of mutant

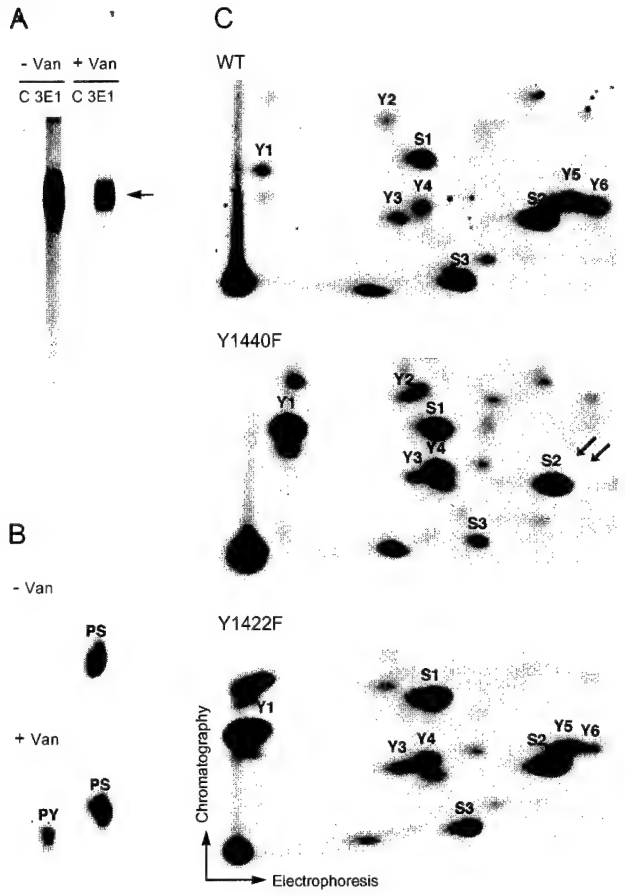


Fig. 7. *In vivo* phosphorylation of the β_4 TAM. (**A**) *In vivo* [^{32}P]orthophosphate labeling of β_4. Rat 804G cells expressing human β_4 were labeled *in vivo* with [^{32}P]orthophosphate and then either left untreated (–Van) or treated with 500 μM vanadate for 10 min (+Van). After extraction with RIPA buffer, the samples were immunoprecipitated with rabbit anti-mouse IgG (C) or the anti-human β_4 monoclonal antibody (3E1) and separated by SDS–PAGE. (**B**) Phosphoamino acid analysis of *in vivo* labeled β_4. The ^{32}P-labeled β_4 bands of (A) were subjected to phosphoamino acid analysis. The top panel shows the phosphoamino acid analysis of *in vivo* labeled β_4 from untreated cells (–Van), the bottom panel that from vanadate treated cells (+Van). Identical amounts of radioactivity were loaded on the two TLC plates. (**C**) Mapping of β_4 tyrosine residues phosphorylated *in vivo*. Rat 804G cells expressing either the human wild-type β_4 subunit or the mutant subunits Y1422F or Y1440F were labeled *in vivo* with [^{32}P]orthophosphate, treated with 500 μM vanadate for 10 min and immunoprecipitated with the anti-human β_4 antibody 3E1. After separation by SDS–PAGE, the radioactive bands corresponding to recombinant β_4 polypeptides were subjected to V8 protease digestion and the resulting phosphopeptides were separated by two-dimensional TLC. The top panel shows the map of wild-type β_4 (WT), the middle panel the map of mutant Y1440F (Y1440F) and the bottom panel the map of mutant Y1422F (Y1422F). Phosphoamino acid analysis indicated that the peptides S1–S3 contain exclusively phosphoserine and Y1–Y6 exclusively phosphotyrosine. Arrows point to the position of radioactive phosphopeptides affected by the Y1440F mutation.

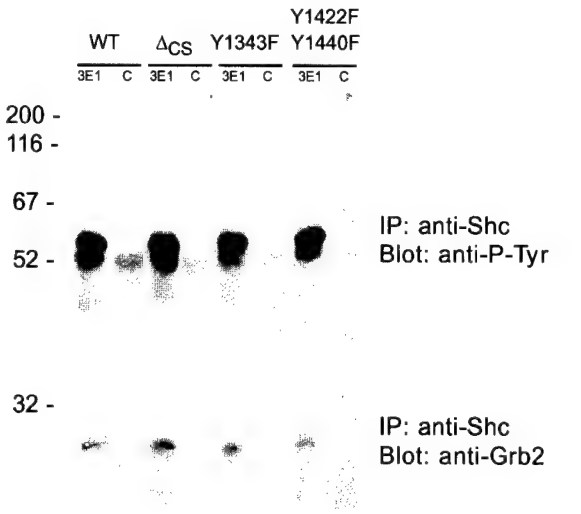


Fig. 8. Shc activation by recombinant β_4 subunits carrying mutations at the TAM sequence. Rat 804G cells expressing the wild-type human β_4 subunit (WT) or mutant versions lacking the entire Connecting Segment (Δ_{CS}) or carrying phenylalanine substitutions in the TAM (Y1422F/Y1440F) or outside the TAM (Y1343F) were incubated for 10 min with anti-human β_4 (3E1)- or anti-MHC (C)-coated beads and extracted. Equal amounts of total proteins were immunoprecipitated with rabbit anti-Shc serum. The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20 (top panel) or with monoclonal anti-Grb2 antibody (bottom panel).

subunits Y1422F and Y1440F. As shown in Figure 7C (middle panel), the replacement of Tyr1440 with phenylalanine caused the disappearance of peptides Y5 and Y6. The simultaneous disappearance of peptides Y5 and Y6 as a consequence of a single point mutation and their similar migration indicate that these peptides are closely related and that both contain Tyr1440. We also observed that the map derived from the Y1440F mutant subunit contained a number of novel peptides and that peptides Y1 and Y4 were more intensely radioactive than in wild-type β_4. Presumably these events are a consequence of compensatory phosphorylation. The substitution of Tyr1422 with phenylalanine caused a reduction in the intensity of only a couple of phosphopeptides (Figure 7C, bottom panel). Also in this case we noticed compensatory phosphorylation (see, for example, peptide Y1). In contrast, the replacement of Tyr1343 with phenylalanine did not result in modification of any phosphopeptide (data not shown). We conclude that the β_4 tail is phosphorylated *in vivo* at multiple tyrosine residues: the C-terminal element of the TAM corresponds to one of the major sites of phosphorylation, while its N-terminal element may correspond to a minor one.

Activation of Shc by $\alpha_6\beta_4$ is not affected by mutations at the β_4 TAM

The role of the β_4 TAM in activation of the Shc/Grb2 pathway was examined using recombinant β_4 subunits carrying either a deletion of the Connecting Segment or phenylalanine substitutions in the β_4 TAM. Rat 804G cells expressing human wild-type or mutant β_4 subunits were incubated with beads coated with either the anti-human β_4 antibody 3E1 or the control anti-MHC antibody W6.32. The samples were immunoprecipitated with anti-Shc antibodies and probed with either anti-P-Tyr or anti-Grb2 antibodies. As shown in Figure 8 (top panel), β_4 subunits with a double mutation in the TAM, a single phenylalanine substitution outside the TAM but within the Connecting Segment or a complete deletion of the Connecting Segment mediated tyrosine phosphorylation of Shc as efficiently as wild-type β_4. In all cases tyrosine phosphorylation of Shc resulted in recruitment of Grb2 (Figure 8, bottom panel).

Re-probing of the blot with the anti-human β_4 monoclonal antibody 450-9D indicated that all the mutant subunits had formed a specific complex with Shc upon stimulation with anti-β_4 beads (data not shown). These results indicate that the β_4 TAM sequence and the entire Connecting Segment are not required for linking $\alpha_6\beta_4$ to Shc and Grb2.

Phosphorylation of the β_4 TAM mediates association of the $\alpha_6\beta_4$ integrin with the cytoskeleton

Previous results indicated that association of the $\alpha_6\beta_4$ integrin with the cytoskeleton and consequent assembly of hemidesmosomes require a specific segment of the β_4 tail (Spinardi *et al.*, 1993, 1995). Since the β_4 TAM is part of this segment and selective inhibition of β_4 phosphorylation with the tyrosine kinase inhibitor herbimycin correlates with inhibition of hemidesmosome assembly (A.Pepe, F.Mainiero and F.G.Giancotti, unpublished results), we asked if phosphorylation of the β_4 TAM played a role in association of the integrin with the hemidesmosomal cytoskeleton. As the $\alpha_6\beta_4$ integrin incorporated in hemidesmosomes is largely resistant to extraction in non-ionic detergents (Spinardi *et al.*, 1993), we examined the Triton X-100 solubility of recombinant β_4 subunits carrying phenylalanine substitutions in the TAM. The result of this experiment indicated that the wild-type β_4 subunit and the control mutant subunit Y1343F, which carries a mutation outside the TAM, are associated predominantly with the Triton X-100-insoluble fraction. In contrast, the mutant subunit Y1422F was equally distributed in the detergent-soluble and -insoluble fractions and the mutant protein Y1440F was exclusively associated with the soluble fraction (Figure 9A). The mutant protein Y1422F/Y1440F was also recovered exclusively from the soluble fraction (data not shown). These results indicate that phosphorylation of the β_4 TAM is important for association of $\alpha_6\beta_4$ with the detergent-insoluble cytoskeleton.

We next examined the subcellular localization of the phenylalanine mutant β_4 subunits by immunofluorescence. Immunostaining with the 3E1 monoclonal antibody showed that wild-type human β_4 is in part diffusely distributed on the plasma membrane and in part concentrated at the basal cell surface within punctate, 'Swiss-cheese-like' structures corresponding to hemidesmosomes (Figure 9B, panel a; Spinardi *et al.*, 1993, 1995). In accordance with previous results, treatment with Triton X-100 prior to fixation eliminated the diffuse staining associated with the plasma membrane, but rendered more evident the 'Swiss-cheese-like' staining of hemidesmosomes (panel d). Cells expressing the control mutant subunit Y1343F, which carries a single phenylalanine substitution outside the connecting segment, displayed a staining pattern identical to that of control cells, indicating that this recombinant molecule is correctly targeted to hemidesmosomes (data not shown). In contrast, the staining pattern generated by the 3E1 antibody in cells expressing the mutant subunit Y1422F was mostly diffuse and associated with the plasma membrane (panel b). Although punctate staining could be detected in cells treated with Triton X-100 before fixation, this staining was much more scarce than that in control cells expressing wild-type β_4 and 'Swiss-cheese-like' structures were never observed (panel e). This indicates that association of the mutant subunit Y1422F with hemidesmosomes is impaired as compared with that of wild-type β_4. Finally, the 3E1 antibody generated only diffuse staining of the plasma membrane in cells expressing the mutant subunit Y1440F (panel c). Notably, virtually all staining was suppressed if the cells were treated with Triton X-100 prior to immunostaining (panel f). Identical results were obtained from an analysis of the subcellular localization of mutant subunit Y1422F/Y1440F (data not shown). Thus mutant β_4 subunits carrying either a single phenylalanine permutation at position 1440 or a double substitution at positions 1422 and 1440 can be detected at the cell surface, but not in hemidesmosomes. Taken together, these findings indicate that stable association of $\alpha_6\beta_4$ with the cytoskeleton at hemidesmosomes requires phosphorylation of both elements of the β_4 TAM.

Discussion

Although observations made in the past two decades point to a pivotal role of the extracellular matrix in controlling gene expression (Adams and Watt, 1993), the question of how integrins transduce signals at the plasma membrane level has remained in large part unsolved, despite intensive investigation. In this study we have examined the mechanism of signal transduction by the $\alpha_6\beta_4$ integrin. Our results indicate that ligand or antibody binding to $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit and suggest that this event is mediated by a protein tyrosine kinase associated with the integrin. The results of phosphopeptide mapping and mutagenesis experiments indicate that the β_4 cytoplasmic domain is phosphorylated at multiple sites: one site, which corresponds to a bidentate TAM similar to those found in several immune receptors, mediates association of $\alpha_6\beta_4$ with the cytoskeleton of hemidesmosomes, while one or more distinct sites are involved in sequential recruitment of the adaptor molecules Shc and Grb2.

The mechanism of signaling by $\alpha_6\beta_4$ suggested by our results incorporates elements of other receptor systems, such as the recruitment of Shc and Grb2, as well as unique features, such as association with the hemidesmosomal cytoskeleton. Like many cytokine and immune receptors (Kishimoto *et al.*, 1994; Weiss and Littman, 1994), $\alpha_6\beta_4$ lacks an intracellular catalytic domain and relies on its association with a cytoplasmic tyrosine kinase for signal transduction. As tyrosine phosphorylation of β_4 can be triggered by adhesion to a laminin 5 matrix, as well as by antibody-mediated cross-linking, but not by soluble antibodies to $\alpha_6\beta_4$, it is likely that dimerization or oligomerization of the integrin is required either for activating the associated tyrosine kinase or for bringing it into close proximity to its target sequences in the β_4 tail. The identity of the tyrosine kinase associated with $\alpha_6\beta_4$ remains to be determined, but the selective ability of *src* family kinases to induce β_4 phosphorylation in co-transfection experiments (A.Curatola and F.G.Giancotti, unpublished results), together with previous observations indicating that the T cell and B cell receptor TAMs are phosphorylated by *src* family kinases (Weiss and Littman, 1994), suggest that $\alpha_6\beta_4$ may be associated with a *src* family member. The observation that the β_4 subunit can be phosphorylated on

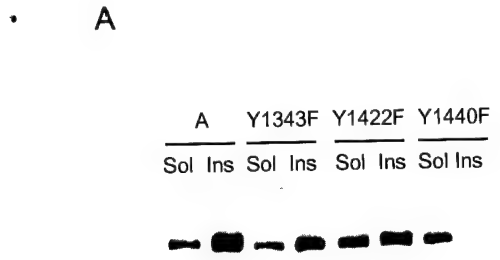
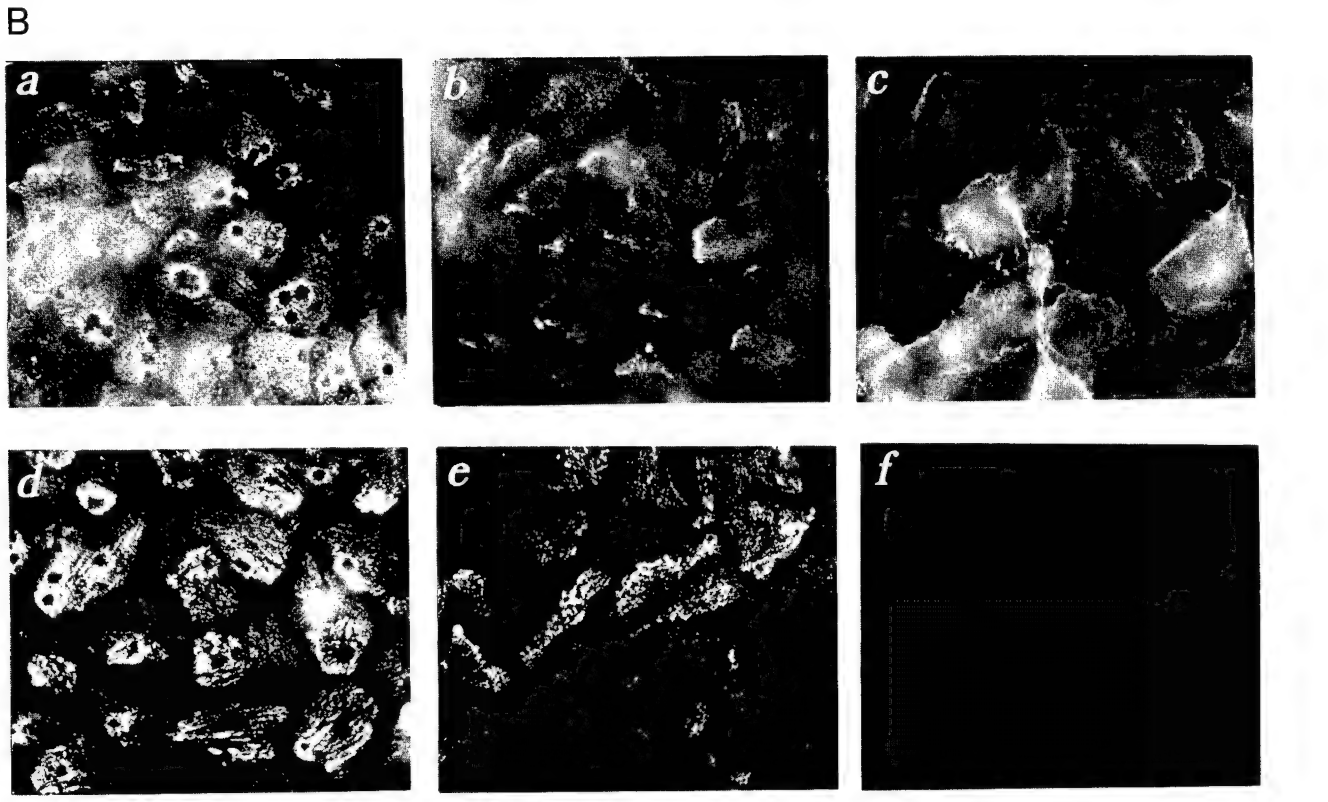


Fig. 9. Phenylalanine replacements in the β_4 TAM interfere with incorporation of $\alpha_6\beta_4$ in hemidesmosomes. (**A**) Triton X-100 solubility of wild-type and mutant β_4 subunits. Triton X-100-soluble (Sol) and -insoluble (Ins) cell fractions were derived from rat 804G cells expressing the human wild-type β_4 subunit (Clone A) or the indicated phenylalanine substituted subunits (Y1343F, Y1422F and Y1440F). After immunoprecipitation with the 3E1 antibody, the samples were probed by immunoblotting with rabbit anti-β_4 serum. In this experiment a smaller number of cells was used to generate detergent-soluble and -insoluble fractions from 804G cells expressing the Y1440F mutant. (**B**) Localization of wild-type and mutant β_4 subunits to hemidesmosomes. Rat 804G cells expressing human wild-type β_4 (a and d), the mutant Y1422F (b and e) or the mutant Y1440F (c and f) were plated on coverslips, cultured for 48 h and then either fixed directly with cold methanol for 2 min (a, b and c) or treated with 0.2% Triton X-100 for 5 min prior to fixation (d, e and f). Immunofluorescent staining was performed using the 3E1 antibody followed by FITC-conjugated goat anti-mouse IgG. Identical results were obtained with three independent clonal cell lines of each type.



serine and threonine residues in immune complex kinase assays indicates that $\alpha_6\beta_4$ may also be associated with other kinases, highlighting the complexity of $\alpha_6\beta_4$ function.

We have observed that ligation of $\alpha_6\beta_4$ results in its association with the adaptor protein Shc. This molecule contains two distinct domains capable of interacting with tyrosine-phosphorylated sequences: an N-terminal PID (Kavanaugh and Williams, 1994; Bork and Margolis, 1995) and a C-terminal SH2 domain (Pellicci *et al.*, 1992). The GST fusion protein binding experiments of this study suggest that both Shc domains can interact independently and directly with the tyrosine-phosphorylated β_4 subunit. Interestingly, the β_4 tail contains two tyrosine-based motifs potentially able to interact with the Shc SH2 domain (Songyang *et al.*, 1994) and three N-X-X-Y motifs which could bind to the Shc PID (Kavanaugh *et al.*, 1995). Although definition of the β_4 sequences involved in interaction with Shc requires further mutagenesis experiments, the present results suggest that the PID and SH2 domains of Shc may bind to β_4 by a cooperative mechanism similar to that described for their binding to the epidermal growth factor receptor (Batzer *et al.*, 1995). As a consequence of its binding to $\alpha_6\beta_4$, Shc is phosphorylated on tyrosine, an event presumably mediated by the kinase associated with $\alpha_6\beta_4$, and then binds to Grb2. Several recent studies have indicated that Grb2 is stably associated

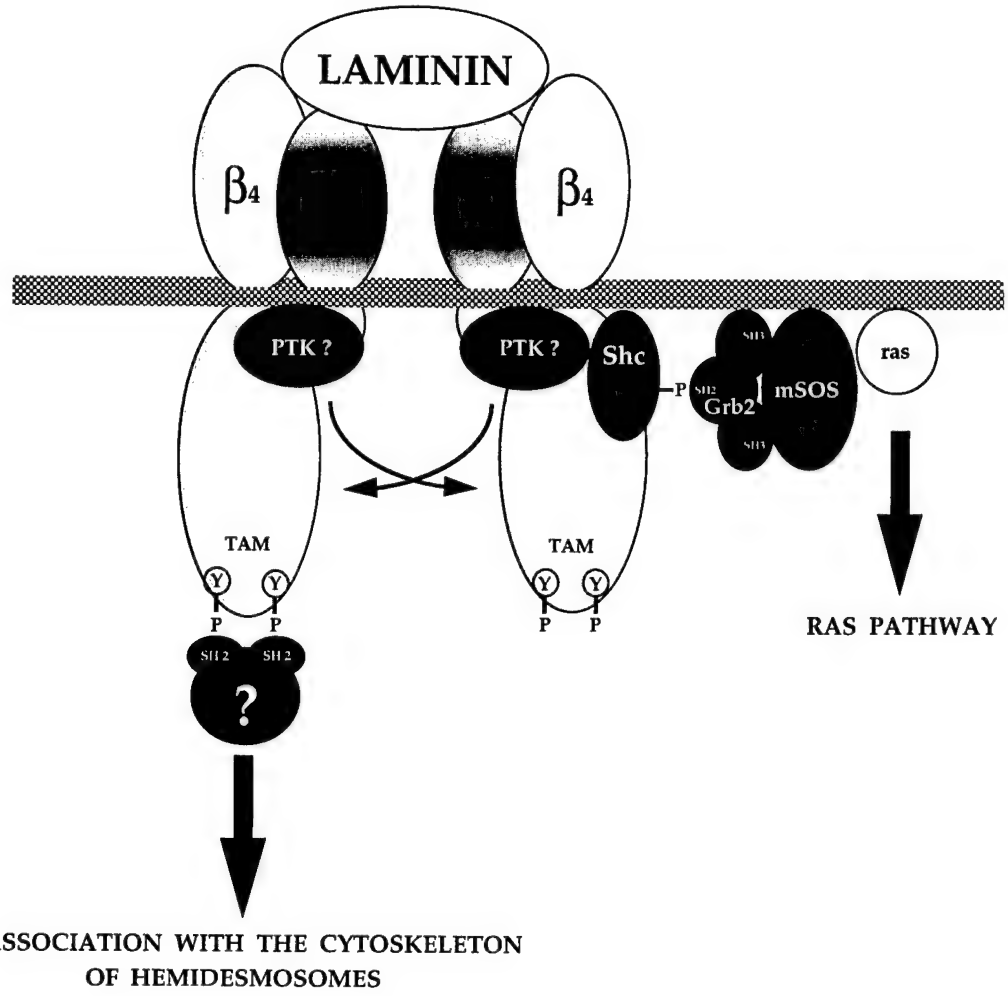


Fig. 10. Schematic model of $\alpha_6\beta_4$ integrin signal transduction. Laminin-induced dimerization or oligomerization of the integrin is followed by activation of an associated protein tyrosine kinase (PTK) that phosphorylates the β_4 tail at multiple residues. Phosphorylation at the β_4 TAM results in association of the integrin with the hemidesmosomal cytoskeleton. The identity of the SH2–SH2 signaling component that we hypothesize interacts with the phosphorylated β_4 TAM and mediates cytoskeletal association is unknown. Distinct β_4 tyrosine phosphorylation motifs mediate the recruitment of Shc. Subsequent tyrosine phosphorylation is likely to be mediated by the tyrosine kinase associated with the integrin. The Grb2–mSOS complex binds to tyrosine-phosphorylated Shc and is thereby recruited to the plasma membrane, where it can activate *ras*.

with the *ras* GTP exchanger mSOS (Schlessinger, 1994; Pawson, 1995). However, while in unactivated cells the complex is confined to the cytoplasm, in stimulated cells it is recruited to the activated receptors and therefore translocated to the plasma membrane, where it can activate *ras*. Our results therefore describe a molecular mechanism potentially linking the $\alpha_6\beta_4$ integrin to the *ras* signaling pathway. In the future it will be important to delineate the specific intracellular pathways activated by recruitment of Shc and Grb2 to $\alpha_6\beta_4$ and elucidate their effects on cell function.

Binding of laminin 5 to $\alpha_6\beta_4$ integrin plays an essential role in the organization of hemidesmosomes (Spinardi *et al.*, 1995). The results of this study suggest that this function requires phosphorylation of the β_4 TAM. Mutations which prevented tyrosine phosphorylation of the β_4 TAM also suppressed association of $\alpha_6\beta_4$ with hemidesmosomes. Interestingly, the replacement of Tyr1440 had a more drastic effect on $\alpha_6\beta_4$ function than mutation of Tyr1422, indicating that phosphorylation of the C-terminal tyrosine may be sufficient for partial functioning of the β_4 TAM. It must be noted that tyrosine phosphorylation of β_4 occurs only transiently in response to ligation of $\alpha_6\beta_4$. In fact, virtually no tyrosine phosphorylated β_4 is detected in stably adherent cells, in which the majority of $\alpha_6\beta_4$ is in hemidesmosomes. Thus it is unlikely that the formation of hemidesmosomes depends on a stable interaction mediated by tyrosine-phosphorylated β_4 TAM. Instead, it is possible that the β_4 TAM is primarily involved in transducing a signal required for hemidesmosome assembly.

What is the nature of this signal? The TAM was originally identified as a common motif present in several immune receptors (Reth, 1989). In the TCR system, as a result of simultaneous binding of the TCR α/β heterodimer and co-receptor CD4 to the peptide-bearing MHC molecule, *lck* comes into close proximity to and phosphorylates the TAMs present in the multichain invariant CD3 complex. Phosphorylation of ζ chain TAMs provides a template for binding of the tyrosine kinase ZAP70 involved in subsequent downstream signaling events (Weiss and Littman, 1994). It is possible that the mechanism by which phosphorylation of the β_4 TAM regulates cytoskeletal assembly also involves binding to an SH2

domain-containing protein. The tyrosine kinases ZAP70 and *syk* contain two tandem SH2 domains through which they bind to the phosphorylated TAMs of the T cell and B cell receptors respectively (Weiss and Littman, 1994). These molecules, however, are restricted to the immune system. In addition, the spacing between Tyr1422 and Tyr1440 in β_4 is larger than the distance between the tyrosines in other TAMs. These observations raise the possibility that the β_4 TAM has a distinct binding specificity. To prove this model it will be necessary to identify the protein kinase or adaptor interacting with the β_4 TAM.

In sum, the results of this study suggest a model of signal transduction by $\alpha_6\beta_4$ integrin that involves a number of sequential steps (Figure 10). We hypothesize that upon binding to a multivalent extracellular matrix ligand $\alpha_6\beta_4$ dimerizes or oligomerizes on the plasma membrane, thereby activating an associated intracellular tyrosine kinase and/or juxtaposing it to its target sequences in the β_4 tail. The phosphorylated β_4 subunit then interacts with Shc and Grb2, as well as with molecules involved in assembly of hemidesmosomes. These two functions appear to be mediated by distinct motifs, because mutations in the β_4 TAM selectively interfere with association of the integrin with the hemidesmosomal cytoskeleton.

The $\alpha_6\beta_4$ signaling mechanism proposed here appears to be especially suited to allow fine tuning of distinct intracellular functions in response to diverse environmental cues. The level of phosphorylation of distinct receptor sites may diverge substantially depending on the nature of the extracellular ligand (Sloan-Lancaster *et al.*, 1994). Thus it is possible that the β_4 TAM and the distinct site involved in binding to Shc are differentially phosphorylated depending on the specific laminin isoform encountered by the cell or its oligomerization state. In addition, the level of phosphorylation of each site may vary with the cell type and its state of differentiation. This potential mechanism is attractive because it would allow a differential regulation of the *ras* pathway and assembly of hemidesmosomes depending on the matrix and cellular context. It is possible that the growth advantage of squamous carcinoma cells is at least in part related to overexpression of $\alpha_6\beta_4$ in these cells (Kimmel and Carey, 1986; Savoia *et al.*, 1993; Tennenbaum *et al.*, 1993) and to its ability to link to the *ras* pathway. Squamous carcinoma cells, however, lack well-organized hemidesmosomes (Schenk, 1979), suggesting that the signals responsible for hemidesmosome assembly may be defective in these cells. Thus these cells may represent an extreme example of the divergent regulation of $\alpha_6\beta_4$-mediated signals.

Finally, the signal transduction mechanism described in this paper provides a rational basis for the effects of $\alpha_6\beta_4$ on morphogenesis and tumor progression. Although it is likely that the intracellular signals elicited by laminin binding to $\alpha_6\beta_4$ are unique, future studies will undoubtedly reveal the extent of signaling overlap between various integrins. The recent observation that $\alpha_v\beta_3$ associates with insulin receptor substrate 1 in insulin-stimulated cells (Vuori and Ruoslahti, 1994) suggests that an additional level of complexity in integrin signaling may result from interaction between growth factor- and adhesion-dependent pathways. In this context, the results of this study represent a first step toward understanding the mechanisms of signal transduction by integrins.

Materials and methods

Antibodies

The monoclonal antibody 3E1, reacting with the extracellular portion of human β_4, and the rabbit polyclonal antiserum to the C-terminal peptide of β_4 have been described previously (Giancotti *et al.*, 1992). The anti-β_4 monoclonal antibody 450-9D and 450-11A have also been previously characterized (Kennel *et al.*, 1990). The monoclonal antibody AIIB2 binds to the extracellular portion of the human β_1 subunit (Werb *et al.*, 1989). The anti-MHC monoclonal antibody W6.32 reacts with human and cultured rat cells (Kahn-Perles *et al.*, 1987). The rabbit polyclonal anti-P-Tyr serum 72 was produced according to published procedures (Kamps and Sefton, 1988). The monoclonal anti-P-Tyr antibody 4G10 was from UBI (Lake Placid, NY). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum 410 was obtained by immunizing a rabbit with a GST fusion protein containing the SH2 domain of the protein (Batzer *et al.*, 1995). The monoclonal antibody EL-6 recognizes an epitope in the SH2 domain of Grb2.

Constructs and transfections

All eukaryotic expression constructs were assembled in the CMV promoter-based vector pRC-CMV (Invitrogen Corp., San Diego, CA). The plasmids encoding the wild-type and tail-less human β_4 subunits have been previously described (Spinardi *et al.*, 1993). To generate the construct pCMV-β_4 Δ1314–1486, which directs expression of a truncated β_4 subunit lacking the Connecting Segment (Δ_{CS}), we employed the polymerase chain reaction (PCR) to engineer a DNA fragment encoding β_4 residues 1315–1485 flanked by *Sac*I (5′-end) and *Not*I (3′-end) sites. The 4.8 kb *Hin*dIII–*Xba*I fragment of β_4 was subcloned into pSL1180 (Pharmacia, Piscataway, NJ), thus generating pSL1180-β_4, and the 5.2 kb *Not*I–*Sac*I fragment of this plasmid was ligated to the PCR-generated β_4 fragment. The 4.3 kb *Bsp*EI–*Xba*I fragment of the resulting plasmid was finally ligated to the 6.3 kb *Xba*I–*Bsp*EI fragment of pCMV-β_4. Phenylalanine substitutions were introduced into β_4 using the Altered Sites *in vitro* mutagenesis system (Promega, Madison, WI). Correctness of all the constructs was verified by sequencing. Rat bladder carcinoma 804G cells were transfected with the various expression constructs and pSV-neo as previously described (Giancotti *et al.*, 1994). Clones expressing comparable levels of each recombinant β_4 polypeptide were selected by FACS analysis. Immunoprecipitation of cells labeled metabolically with [^{35}S]methionine was used to verify correct assembly of the recombinant β_4 polypeptides with the endogenous α_6 subunit (Spinardi *et al.*, 1993).

GST fusion proteins encoding the murine Shc PID (residues 1–209) and SH2 domains were expressed and purified on glutathione–agarose beads as previously described (Blaikie *et al.*, 1994).

Biochemical methods

To obtain selective ligation of $\alpha_6\beta_4$ in the absence of any co-stimulus, the cells were serum starved, detached with 10 mM ethylenediamine tetraacetate (EDTA) and then resuspended at 20×10^6/ml. Aliquots (200 μl) of this cell suspension were incubated at 37°C and either stimulated with 1.8×10^8 polystyrene sulfate latex beads (2.5 μm diameter; IDC, Portland, OR) coated with the 3E1 or the control W6.32 monoclonal antibody (400 μg/ml) for the indicated times or left untreated. To obtain engagement of $\alpha_6\beta_4$ by a physiological ligand, the cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes (Spinardi *et al.*, 1995) for the indicated times. At the end of the incubation the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS) or lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetate (EGTA) (all from Sigma, St Louis, MO).

To examine the detergent solubility of phenylalanine mutant β_4 subunits, subconfluent monolayers of the various clones were extracted on ice with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Triton X-100 and protease inhibitors for 5 min. The detergent-soluble fraction was recovered and the insoluble cytoskeletons were washed and then extracted with RIPA buffer and protease inhibitors. Detergent-soluble and -insoluble fractions derived from the same sample were directly compared.

Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti *et al.*, 1992). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

For binding studies, rat 804G cells expressing the human wild-type β_4 subunit were serum starved and treated with 100 μM sodium orthovanadate plus 3 mM H_2O_2 or stimulated in suspension with anti-β_4 beads for 10 min at 37°C. After extraction in SDS buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS) with protease inhibitors, the lysates were heated for 5 min at 80°C, sonicated and diluted with 9 vol. lysis buffer. Glutathione–agarose beads carrying the GST fusion proteins were incubated with the denatured lysates (10 μg fusion protein/1 mg total proteins) for 2 h at 4°C, washed and boiled in SDS–PAGE sample buffer. Samples were separated by SDS–PAGE and analyzed by immunoblotting with polyclonal anti-β_4 antibodies.

For immune complex kinase assay, subconfluent cell monolayers were extracted with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Brij 96 and phosphatase and protease inhibitors. After clarification, the extracts were immunoprecipitated as described above. The affinity beads were washed extensively with the extraction buffer without phosphatase inhibitors and then equilibrated in kinase buffer (10 mM Tris, pH 7.4, 10 mM $MnCl_2$, 20 mM *p*-nitrophenylphosphate). The kinase reaction was initiated by adding 50 μl kinase buffer containing 20 μCi [γ-^{32}P]ATP (4500 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) to the beads and continued at 30°C for 30 min. The reaction was stopped by boiling the samples for 5 min in SDS–PAGE sample buffer.

Phosphoamino acid analysis was performed as described by Boyle *et al.* (1991). ^{32}P-Labeled β_4 was eluted from fixed polyacrylamide gels and precipitated with 20% trichloroacetic acid. ^{32}P-Labeled peptides were scraped off TLC plates, eluted in pyridine and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC electrophoresis in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid) for the first dimension (1.5 kV, 40 min) and in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension (1.5 kV, 30 min). Non-radioactive standards were detected by ninhydrin staining, while radiolabeled phosphoamino acids were observed by autoradiography.

Phosphopeptide mapping was performed essentially as described by Boyle *et al.* (1991). Cells were labeled metabolically with [^{32}P]orthophosphate (3 mCi/ml; ICN) for 3 h and then either treated with 500 μM sodium orthovanadate and 3 mM H_2O_2 for 10 min at 37°C or left untreated. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β_4 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μl 50 mM phosphate buffer, pH 7.8, with 25 μg *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (1.5 kV, 50 min) and in the second by ascending chromatography in Phospho Chromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid).

Immunofluorescence

Cells were either fixed directly with cold methanol for 2 min or treated with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min on ice prior to fixation with methanol. Immunostaining with 3E1 antibody was performed as previously described (Spinardi *et al.*, 1993, 1995). Secondary antibodies were species-specific. Samples were examined with a Zeiss Axiophot Fluorescent Microscope.

Acknowledgements

We thank C.Damsky, E.Engvall, S.J.Kennel and A.Sacchi for antibodies, A.Batzer and B.Margolis for helpful suggestions and Shc cDNAs, J.Sap for critical comments on the manuscript and M.Yeon for expert technical assistance. This work was supported by PHS grant R01-CA58976, DAMD grant 17-94-J4306 and PHS core support grant P30-CA16087. F.M. was supported by a fellowship from the Associazione Italiana per la Ricerca sul Cancro. F.G.G. is the recipient of an award from the Lucille P.Markey Charitable Trust.

References

Adams,J.C. and Watt,F.M. (1993) Regulation of development and differentiation by extracellular matrix. *Development*, **117**, 1183–1198.

Batzer,A.G., Blaikie,P., Nelson,K., Schlessinger,J. and Margolis,B. (1995) The PI domain of Shc binds an LXNPXY motif on EGF receptor. *Mol. Cell. Biol.*, **15**, 4403–4409.

Blaikie,P., Immanuel,D., Wu,J., Li,N., Yajnik,V. and Margolis,B. (1994) A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J. Biol. Chem.*, **269**, 32031–32034.

Bockholt,S.M. and Burridge,K. (1993) Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. *J. Biol. Chem.*, **268**, 14565–15567.

Bork,P. and Margolis,B. (1995) A phosphotyrosine interaction domain. *Cell*, **80**, 693–694.

Boyle,W.J., Van Der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Burridge,K., Turner,C.E. and Romer,L.H. (1992) Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.*, **119**, 893–903.

Carter,W.G., Kaur,P., Gil,S.G., Gahr,P.J. and Wayner,E.A. (1990) Distinct functions for integrins $\alpha_3\beta_1$ in focal adhesions and $\alpha_6\beta_4$/Bullous Pemphigoid Antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.*, **111**, 3141–3154.

Chong,L.D., Traynor-Kaplan,A., Bokoch,G.M. and Schwartz,M.A. (1994) The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507–513.

DiPersio,C.M., Jackson,D.A. and Zaret,K.S. (1991) The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mol. Cell. Biol.*, **11**, 4405–4414.

Einheber,S., Milner,T.A., Giancotti,F.G. and Salzer,J.L. (1993) Axonal regulation of Schwann cell integrin expression suggests a role for $\alpha_6\beta_4$ in myelination. *J. Cell Biol.*, **123**, 1223–1236.

Enenstein,J. and Kramer,R.H. (1994) Confocal microscopic analysis of integrin expression on the microvasculature and its sprouts in the neonatal foreskin. *J. Invest. Dermatol.*, **103**, 381–386.

Engvall,E. (1993) Laminin variants: why, where and when? *Kidney Int.*, **43**, 2–6.

Giancotti,F.G. and Mainiero,F. (1994) Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta Rev. Cancer*, **1198**, 47–64.

Giancotti,F.G. and Ruoslahti,E. (1990) Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell*, **60**, 849–859.

Giancotti,F.G., Stepp,M.A., Suzuki,S., Engvall,E. and Ruoslahti,E. (1992) Proteolytic processing of endogenous and recombinant β_4 integrin subunit. *J. Cell Biol.*, **118**, 951–959.

Giancotti,F.G., Spinardi,L., Mainiero,F. and Sanders,R. (1994) Expression of heterologous integrin genes in cultured eukaryotic cells. *Methods Enzymol.*, **245**, 297–316.

Guan,J.-L. and Shalloway,D. (1992) Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature*, **358**, 690–692.

Hanks,S.K., Calalb,M.B., Harper,M.C. and Patel,S.K. (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl Acad. Sci. USA*, **89**, 8487–8491.

Hogervorst,F., Kuikman,I., von dem Borne,A.E.G.,Jr and Sonnenberg, A. (1990) Cloning and sequence analysis of β_4 cDNA: an integrin subunit that contains a unique 118 kDa cytoplasmic domain. *EMBO J.*, **9**, 745–770.

Huang,M.-M., Lipfert,L., Cunningham,M., Brugge,J.S., Ginsberg,M.H. and Shattil,S.J. (1993) Adhesive ligand binding to integrin $\alpha_{II}\beta_3$ stimulates tyrosine phosphorylation of novel protein substrates before the phosphorylation of pp125^{FAK}. *J. Cell Biol.*, **122**, 473–483.

Hynes,R.O. (1992) Integrins: versatility, modulation and signaling in cell adhesion. *Cell*, **69**, 11–25.

Juliano,R.L. and Haskill,S. (1993) Signal transduction from the extracellular matrix. *J. Cell Biol.*, **120**, 577–585.

Kahn-Perles,B., Boyer,C., Arnold,B., Sanderson,A.R., Ferrier,P. and Lemonnier,F. (1987) Acquisition of HLA class I W6/32 defined antigenic determinant by heavy chains from different species following association with bovine β_2-microglobulin. *J. Immunol.*, **138**, 2190–2196.

Kajiji,S., Tamura,R.N. and Quaranta,V. (1989) A novel integrin ($\alpha_E\beta_4$)

from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.*, **8**, 673–680.

Kamps,M.P. and Sefton,B.M. (1988) Identification of novel polypeptide substrates of the v-src, v-yes, v-fps, v-ros, v-erb-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene*, **2**, 305–315.

Kavanaugh,W.M. and Williams,L.T. (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science*, **266**, 1862–1865.

Kavanaugh,W.M., Turck,C.W. and Williams,L.T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science*, **268**, 1177–1179.

Kennel,S.J. *et al.* (1990) Second generation monoclonal antibodies to the human integrin $\alpha_6\beta_4$. *Hybridoma*, **9**, 243–255.

Kimmel,K.A. and Carey,T.E. (1986) Altered expression in squamous carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.*, **46**, 3614–3623.

Kishimoto,T., Taga,T. and Akira,S. (1994) Cytokine signal transduction. *Cell*, **76**, 253–262.

Lipfert,L., Haimovich,B., Shaller,M.D., Cobb,B.S., Parsons,J.T. and Brugge,J.S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase $pp125^{FAK}$ in platelets. *J. Cell Biol.*, **119**, 905–912.

McNamee,H.M., Ingber,D.E. and Schwartz,M.A. (1992) Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-inositol lipid breakdown. *J. Cell Biol.*, **121**, 673–678.

Mercurio,A.M. (1990) Laminin: multiple forms, multiple receptors. *Curr. Opin. Cell Biol.*, **2**, 845–849.

Pawson,T. (1995) Protein modules and signalling networks. *Nature*, **373**, 573–580.

Pelicci,G. *et al.* (1992) A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, **70**, 93–104.

Reth,M. (1989) Antigen receptor tail clue. *Nature*, **338**, 383–384.

Roskelley,C.D., Desprez,P.Y. and Bissell,M.J. (1994) Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl Acad. Sci. USA*, **91**, 12378–12382.

Ruoslahti,E. (1991) Integrins. *J. Clin. Invest.*, **87**, 1–5.

Savoia,P., Trusolino,L., Pepino,E., Cremona,O. and Marchisio,P.C. (1993) Expression and topography of integrins and basement membrane proteins in epidermal carcinomas. *J. Invest. Dermatol.*, **101**, 352–358.

Schenk,P. (1979) The fate of hemidesmosomes in laryngeal carcinoma. *Arch. Oto-Rhino-Laryngol.*, **222**, 187–198.

Schlaepfer,D.D., Hanks,S.K., Hunter,T. and van der Geer,P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786–791.

Schlessinger,J. (1994) SH2/SH3 signaling proteins. *Curr. Opin. Genet. Dev.*, **4**, 25–30.

Shaller,M.D., Borgman,C.A., Cobb,B.S., Vines,R.R., Reynolds,A.B. and Parsons,J.T. (1992) $pp125^{FAK}$, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl Acad. Sci. USA*, **89**, 5192–5196.

Shattil,S.J., Haimovich,B., Cunningham,M., Lipfert,L., Parsons,J.T., Ginsberg,M.H. and Brugge,J.S. (1994) Tyrosine phosphorylation of $\alpha_{IIb}\beta_3$ in platelets requires coordinated signaling through integrin and agonist receptors. *J. Biol. Chem.*, **269**, 14738–14745.

Sloan-Lancaster,J., Shaw,A.S., Rothbard,J.B. and Allen,P.M. (1994) Partial T cell signaling: altered phospho-ζ and lack of ZAP-70 recruitment in APL-induced T cell anergy. *Cell*, **79**, 913–922.

Songyang,Z. *et al.* (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767–778.

Songyang,Z. *et al.* (1994) Specific motifs recognized by the SH2 domains of csk, 3BP2, fps/fes, GRB2, HCP, SHC, syk and vav. *Mol. Cell. Biol.*, **14**, 2777–2785.

Spinardi,L., Ren,Y.-L., Sanders,R. and Giancotti,F.G. (1993) The β_4 subunit cytoplasmic domain mediates the interaction of $\alpha_6\beta_4$ integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell*, **4**, 871–884.

Spinardi,L., Einheber,S., Cullen.,T., Milner,T.A. and Giancotti,F.G. (1995) A recombinant tail-less integrin $\alpha_6\beta_4$ subunit disrupts hemidesmosomes, but does not suppress $\alpha_6\beta_4$-mediated cell adhesion to laminins. *J. Cell Biol.*, **129**, 473–487.

Stepp,M.A., Spurr-Michaud,S., Tisdale,A., Elwell,J. and Gipson,I.K. (1990) Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl Acad. Sci. USA*, **87**, 8970–8974.

Suzuki,S. and Naitoh,Y. (1990) Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.*, **9**, 757–763.

Tamkun,J.W., DeSimone,D.W., Fonda,D., Patel,R.S., Buck,C., Horwitz,H.F. and Hynes,R.O. (1986) Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell*, **46**, 271–282.

Tennenbaum,T., Weiner,A.K., Belanjer,A.J., Glick,A.B., Hennings,H. and Yuspa,S.H. (1993) The suprabasal expression of $\alpha_6\beta_4$ integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. *Cancer Res.*, **53**, 4803–4810.

Vuori,K. and Ruoslahti,E. (1994) Association of insulin receptor substrate-1 with integrins. *Science*, **266**, 1576–1578.

Wadsworth,S., Halvorson,M.J. and Coligan,J.E. (1992) Developmentally regulated expression of the β_4 integrin on immature mouse thymocytes. *J. Immunol.*, **149**, 421–428.

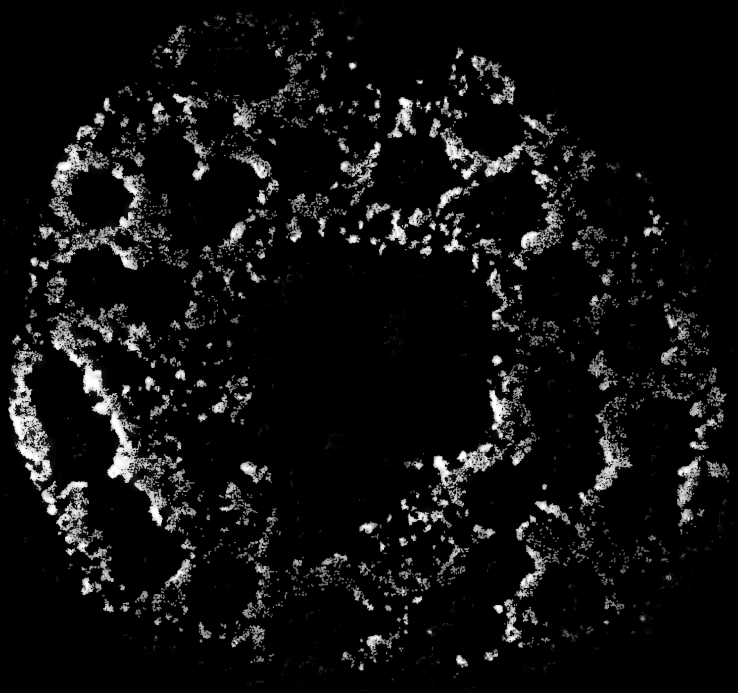
Weiss,A. and Littman,D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263–274.

Werb,Z., Tremble,P.M., Behrendtsen,O., Crowley,E. and Damsky,C.H. (1989) Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.*, **109**, 877–889.

Received on April 24, 1995; revised on June 28, 1995

Journal of Cell Science

VOLUME 109 PART 6 JUNE 1996



The Company of Biologists Limited

INFORMATION ABOUT THE JOURNAL

JOURNAL OF CELL SCIENCE (ISSN 0021-9533)

Subscriptions

Published by The Company of Biologists Limited, Bidder Building, 140 Cowley Road, Cambridge CB4 4DL, UK, and distributed by Virgin Mailing and Distribution, 10 Camptown Road, Irvington, New Jersey 07111-1105, USA.

One volume of 12 parts will be published monthly in 1996.

The subscription price includes air-speeded delivery to most countries.

USA, Canada and Mexico
Institutional: US$1125 Individual: US$185

Rest of the World
Institutional: £645 Individual: £105
(All prices include post and packaging).

Individual rates apply only when payment is made by personal cheque or credit card and is sent direct to the Company, not by way of an agent.

Orders must be accompanied by payment or credit card authorization with details, payable to The Company of Biologists Limited, and sent to the address above. Please contact the above address for prices of back volumes.

Second Class postage paid at Newark, N.J.

Address changes
SUBSCRIBERS send changes to: The Subscription Dept, The Company of Biologists Limited, 140 Cowley Rd., Cambridge CB4 4DL, UK.
POSTMASTER send address changes to: Journal of Cell Science, c/o Virgin Mailing and Distribution, 10 Camptown Road, Irvington, New Jersey 07111-1105, USA.

Journal of Cell Science

Journal of Cell Science publishes critical work over the full range of cell biology. Papers are called for that deal with all aspects of both prokaryotic and eukaryotic cells, the single most important criterion for acceptance being scientific excellence. Short surveys of topical subjects appear in each issue under the general title *Commentary.*

What Journal of Cell Science offers

(i) Wide international circulation
(ii) Fast publication - average 13 weeks from acceptance
(iii) Fast editorial processing
(iv) No page charges
(v) 50 free reprints
(vi) Free colour reproduction (at the editor's discretion).

Rapid publication

Journal of Cell Science now offers **rapid publication** (average 9 weeks from acceptance). The following criteria apply:
(i) the revised manuscript is accompanied by a corresponding word-processor disk
(ii) no colour plates
(iii) author does not require to see proofs
(iv) 50 free reprints only
(v) manuscript conforms to journal style, i.e. no subediting necessary - see Information for contributors.
(vi) Figures are labelled in journal style.

Reagents

Publication of a paper in this journal implies that authors will make available specialized reagents (for example antibodies, DNA probes) to other scientists in the field who require them for bona fide purposes.

For detailed instructions to authors, please see back cover.

Copyright and reproduction

Disclaimer to authors

All authors should note that responsibility for:

(i) the accuracy of statements of fact;
(ii) the authenticity of scientific findings or observations;
(iii) expressions of scientific or other opinion; and
(iv) any other material published in this Journal

rests solely with the author(s) of the article in which such statements etc appear, and that no responsibility for such matters is assumed by the Journal, its owners, publishers or staff (including referees).

Disclaimer to readers

All statements of fact, scientific findings and observations, expressions of scientific or other opinion, and other material published in this Journal, are the sole responsibility of the author(s). No responsibility for such matters on the part of the Journal, its owners, publishers or staff is assumed or to be implied.

Cover picture

Confocal laser scanning image of a rat bladder epithelial 804G cell expressing a recombinant human β_4 integrin subunit. The recombinant β_4, which combines with endogenous α_6, is stained with specific antibodies (green). The image is an optical section corresponding to the basal cell surface viewed from the top. The $\alpha_6\beta_4$ integrin is concentrated in granular structures corresponding to hemidesmosomes. The staining pattern is defined as 'swiss-cheese'-like because the hemidesmosomes are excluded from circular areas of the basal cell membrane. See article in this issue by F. G. Giancotti (pp. 1165-1172).

Journal of Cell Science 109, 1165-1172 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JCS4196

COMMENTARY

Signal transduction by the $\alpha_6\beta_4$ integrin: charting the path between laminin binding and nuclear events

Filippo G. Giancotti

Department of Pathology and Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016, USA

(e-mail: giancf01@mcrcr6.med.nyu.edu)

INTRODUCTION

Basement membranes contribute to the organization of tissues and organs and play dynamic roles in tissue morphogenesis, wound repair and cellular trafficking (Paulsson, 1992; Timpl, 1989). The biological effects of basement membranes can in large part be attributed to laminins, a large family of homologous extracellular matrix proteins expressed in a tissue specific fashion (Engvall, 1993). By binding to cell surface integrins, laminins promote cell adhesion and exert profound influences on cell survival, proliferation and differentiation (Mercurio, 1995). It is likely that the effects of laminins on cellular behavior depend on the ability of integrins to participate in intracellular signaling (Clark and Brugge, 1995; Giancotti and Mainiero, 1994; Schwartz et al., 1995). However, the mechanisms by which integrins transduce signals accross the plasma membrane to ultimately affect gene expression are incompletely understood. In this commentary I will focus on the signaling functions of the laminin-binding integrin $\alpha_6\beta_4$. Recent studies have revealed that $\alpha_6\beta_4$ is associated with a cytoplasmic tyrosine kinase and linked to the *ras* signaling pathway by a series of SH2- and SH3-containing adaptor molecules (Mainiero et al., 1995). These results provide a molecular basis to the previously recognized ability of basement membranes to affect gene expression and may serve as a paradigm in future studies focusing on the signal transduction mechanisms of other integrins.

ROLE OF $\alpha_6\beta_4$ IN MORPHOGENESIS AND DEVELOPMENT

The $\alpha_6\beta_4$ integrin is a receptor for various laminin isoforms (Lee et al., 1992; Niessen et al., 1994; Spinardi et al., 1995) and it binds with the highest apparent affinity to laminin 5 and laminin 4 (Spinardi et al., 1995). In accordance with its ligand binding specificity, $\alpha_6\beta_4$ is expressed in cell types which interact with laminin-rich matrices in vivo, most notably epithelial (Kajiji et al., 1989), endothelial (Kennel et al., 1992; Klein et al., 1993) and Schwann cells (Sonnenberg et al., 1990; Einheber, 1993). The expression of $\alpha_6\beta_4$ and its laminin ligands is regulated during embryonic development and differentiation suggesting that $\alpha_6\beta_4$ may have important functions during morphogenesis.

In stratified epithelia, such as the epidermis, the expression of $\alpha_6\beta_4$ is restricted to those keratinocytes which abut the basement membrane and are endowed with proliferative capacity (Kajiji et al., 1989). Keratinocytes withdraw from the cell cycle and begin to differentiate as soon as they leave the basement membrane to migrate to the upper layers of skin (Hall and Watt, 1989) and this process can be replicated in vitro by detaching cultured keratinocytes from their extracellular matrix (Green, 1977) which is particularly rich in laminin 5 (Carter et al., 1991; Rousselle et al., 1991). In addition, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of $\alpha_6\beta_4$ (Kimmel and Carey, 1986; Wolf et al., 1990). The correlation between expression of $\alpha_6\beta_4$ and cell proliferation revealed by these observations suggests that this integrin may provide epithelial cells with a signal important for cell cycle progression.

The $\alpha_6\beta_4$ integrin is abruptly downregulated at the onset of keratinocyte differentiation. This is in striking contrast with the gradual disappearance of other laminin binding integrins during epidermal differentiation (Carter et al., 1990a,b). It is likely that a post-transductional mechanism contributes to the rapid disappearance of $\alpha_6\beta_4$ from differentiating keratinocytes. The cytoplasmic domain of β_4 is cleaved in vitro and in cultured cells by the calcium-dependent protease calpain and there is evidence suggesting that this cleavage occurs also in vivo in the basal layer of epidermis (Giancotti et al., 1992). Notably, calpain digestion results in the release of a portion of the β_4 tail that is predicted to be essential for linkage to the hemidesmosomal cytoskeleton (Spinardi et al., 1993). Since the cytoskeletal interactions mediated by the cytoplasmic domain of β_4 appear to be important for long-term stable adhesion to the basement membrane (Spinardi et al., 1995), it is possible that cleavage of the β_4 tail facilitates detachment from the basement membrane at the onset of differentiation. In addition to regulating calpain activity, calcium is a trigger of epidermal differentiation (Hennings et al., 1980) and its concentration in the cytosol of epidermal cells rises as they differentiate (Menon et al., 1985). The ability of calcium to simultaneously control $\alpha_6\beta_4$-mediated adhesion and differentiation

Key words: $\alpha_6\beta_4$ integrin, Hemidesmosome, Signaling

may thus serve to couple the reversal of adhesion to the onset of differentiation in epidermal cells. Alternatively, the down-regulation of integrin-mediated adhesion to the basement membrane may in itself represent a signal contributing to differentiation, as it is suggested by the observation that detachment per se is a sufficient stimulus for epidermal differentiation (Green, 1977).

Immunohistochemical studies have shown that the $\alpha_6\beta_4$ integrin is expressed in a subset of small vessels (Kennel et al., 1992). This observation may reflect an involvement of $\alpha_6\beta_4$ in angiogenesis. Recent studies on the expression of various integrins during dermal angiogenesis in vivo have indicated that $\alpha_6\beta_4$ is enriched at the tip of endothelial sprouts (Enenstein and Kramer, 1994), where it colocalizes with laminins (Jerdan et al., 1991). Furthermore, the prototypic angiogenic factor FGF-2 upregulates the expression of $\alpha_6\beta_4$ in capillary endothelial cells cultured in vitro (Klein et al., 1993). Thus, it is possible that ligation of $\alpha_6\beta_4$ at the growing tip of new vessels provides the endothelial cells with a growth stimulatory signal.

Ensheathment and myelination of axons by Schwann cells in the peripheral nervous system is a process that requires contact with the basement membrane (Bunge et al., 1986; Carey et al., 1986). The $\alpha_6\beta_4$ integrin may be also involved in this process. When undifferentiated, proliferating Schwann cells are co-cultured with neurons under conditions which promote withdrawal from the cell cycle and myelination, they are induced to express de novo significantly high levels of $\alpha_6\beta_4$ (Einheber et al., 1993). In accordance with this observation, β_4 levels also increase during myelination of developing peripheral nerves in vivo (Feltri et al., 1994). The basement membrane that the Schwann cells deposit at the onset of myelination contains the $\alpha_6\beta_4$ ligands laminin 2 and 4 (Sanes et al., 1990; Marinkovich et al., 1992), hence it is likely that $\alpha_6\beta_4$ interacts with these extracellular matrix proteins during myelination. Recent studies are consistent with the hypothesis that the interaction of differentiating Schwann cells with laminins is physiologically important. In particular, the observation that the *dy/dy* mouse, which carries a mutation in the α_2 subunit (LAMA2) gene and therefore lacks both laminin 2 and 4 (Sunada et al., 1994; Xu et al., 1994), develops a form of muscular dystrophy accompanied by peripheral nerve degeneration suggests that $\alpha_6\beta_4$ provides the Schwann cells with a signal important for survival and/or differentiation.

Finally, $\alpha_6\beta_4$ is expressed transiently during T-lymphocyte development. $CD4^-/CD8^-$ pre-T lymphocytes begin to express $\alpha_6\beta_4$ when they enter the thymus and lose the integrin soon after when they become single positive cells (Wadsworth et al., 1992). The thymus contains laminin 2 and 5 (Chang et al., 1993; Jaspars et al., 1993). Thus, it is conceivable that laminin binding to $\alpha_6\beta_4$ participates in regulating the homing or differentiation of pre-T cells. In accordance with this hypothesis, it has been recently reported that T lymphocyte development is defective in the laminin-deficient *dy/dy* mouse (Magner et al., 1995). These observations suggest that $\alpha_6\beta_4$ may be involved in T-cell differentiation.

CHANGES DURING TUMOR PROGRESSION

Neoplastic transformation can profoundly affect the level of expression of the $\alpha_6\beta_4$ integrin, but the direction of the change seems to depend on tumor type. Several studies indicate that $\alpha_6\beta_4$ is expressed at high levels in squamous carcinomas of lung, skin, oral cavity and cervix (Carico et al., 1993; Kimmel and Carey, 1986; Mariani-Costantini et al., 1990; Wolf et al., 1990). In fact, the β_4 subunit was known before its time as a cell surface molecule specifically expressed in squamous carcinomas (Kimmel and Carey, 1986). Although it is not clear if $\alpha_6\beta_4$ is genuinely upregulated in individual squamous carcinoma cells as compared with normal basal keratinocytes, it is evident that the expression of the integrin in squamous cancers is no longer restricted to the basal cell layer, but it extends to several suprabasal layers (Carico et al., 1993; Kimmel and Carey, 1986; Savoia et al., 1993). In addition, $\alpha_6\beta_4$ is diffusely distributed over the entire surface of squamous carcinoma cells (Kimmel and Carey, 1986; Savoia et al., 1993), perhaps because of a defective interaction with the hemidesmosomal cytoskeleton (Shenk, 1979; Bergstraesser et al., 1995). The suprabasal expression of the integrin is likely to reflect an expansion of the stem cell compartment in these malignancies. If this is so, the areas of focal loss of $\alpha_6\beta_4$ detected in some squamous tumors (Jones et al., 1993) may comprise cells which have at least partially differentiated and therefore lost expression of the integrin. To further investigate this hypothesis, it will be important to determine if the $\alpha_6\beta_4$-negative tumor cells are less malignant than those expressing normal or increased levels of the integrin.

Several observations support the notion that the $\alpha_6\beta_4$ integrin is a positive regulator of epidermal cell malignancy. Studies in a well characterized mouse skin carcinogenesis model have indicated that the levels of $\alpha_6\beta_4$ steadily increase during tumor progression (Tennenbaum et al., 1992). In particular, it is significant that $\alpha_6\beta_4$ levels increase only in those skin papillomas which will become carcinomas and that this occurs prior to their full conversion to malignancy and independently of whether the tumors were induced by retroviral transduction or chemical carcinogens (Tennenbaum et al., 1993). In addition, while in normal skin and benign papillomas the β_4 subunit is associated exclusively with the canonical form of α_6, carcinoma cells also express the α_{6B} splicing variant subunit which may enable them to interact more avidly with laminins (Tennenbaum et al., 1995). Finally, it may not be coincidental that carcinoma cells invading the adjacent stroma express on their surface significant amounts of the $\alpha_6\beta_4$ ligand laminin 5 (Pyke et al., 1994, 1995) and carcinoma cells selected for their ability to invade a laminin-rich extracellular matrix display high levels of $\alpha_6\beta_4$ (Dedhar et al., 1993). These observations suggest that increased levels of $\alpha_6\beta_4$ are selected for during tumor progression because they may facilitate the invasive growth of neoplastic epidermal cells.

In apparent contrast with these findings, several observations indicate that $\alpha_6\beta_4$ is downregulated in adenocarcinomas of breast and prostate (Knox et al., 1994; Koukoulis et al., 1991; Natali et al., 1992). It is possible that, while in normal epidermal cells $\alpha_6\beta_4$ functions as a positive regulator of cell growth, in normal breast and prostate cells it contributes to cell cycle withdrawal and differentiation. If this is the case, successful tumor growth would be facilitated by an upregulation of $\alpha_6\beta_4$ in those cell types in which the integrin stimulates cell

growth and by its downregulation in those others in which the integrin promotes differentiation.

STRUCTURE AND CYTOSKELETAL INTERACTIONS

The $\alpha_6\beta_4$ integrin is among integrins unique in structure and subcellular localization. Although the extracellular portion of $\alpha_6\beta_4$ is homologous to that of other known integrins, the cytoplasmic domain of β_4 subunit is over 1,000 amino acids long and bears no homology with the short cytoplasmic domains of other known β subunits (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). It contains towards its C terminus two pairs of type III fibronectin (Fn)-like modules separated by a 142 amino acid sequence (Connecting Segment) which appears to be the target of multiple regulatory mechanisms, including tyrosine phosphorylation (Mainiero et al., 1995) and proteolytic processing (Giancotti et al., 1992). In contrast to β_1 and β_3 integrins which localize to focal adhesions, the $\alpha_6\beta_4$ integrin is found concentrated in hemidesmosomes (Carter et al., 1990a,b; Stepp et al., 1990). This observation indicates that $\alpha_6\beta_4$ interacts with the keratin filament system, and not with the actin cytoskeleton.

The hemidesmosomes are punctuate junctions connecting the basal cells of stratified and complex epithelia to the basement membrane (Schwarz et al., 1990; Legan et al., 1992). At the ultrastructural level they appear as tripartite structures with an electron dense juxta-membranous plaque and an innermost plaque linked to the keratin filaments (Shienvold and Kelly, 1976). The juxta-membranous plaque is connected to the innermost plaque by short filaments. Furthermore, short threads of extracellular matrix called anchoring filaments connect the extracellular aspect of hemidesmosomes to the lamina lucida (Ellison and Garrod, 1984).

The hemidesmosomes are complex structures. Despite the functional importance of hemidesmosomes, relatively little is known about the structure and function of many of their components. Fig. 1 shows a schematic model of the structure and molecular composition of hemidesmosomes. The anchoring filaments contain laminin 5 and the binding of this matrix protein to $\alpha_6\beta_4$ may be the primary determinant in the establishment and maintainance of hemidesmosomes. However, in addition to $\alpha_6\beta_4$, the hemidesmosomes contain another transmembrane protein potentially involved in cell adhesion, the Bullous Pemphigoid Antigen 2 (BPAG 2) (Giudice et al., 1992). Recent results suggest that BPAG2 may interact with the α_6 subunit and perhaps cooperate with $\alpha_6\beta_4$ in mediating cell adhesion to the basement membrane (Hopkinson et al., 1995). All the other known elements of hemidesmosomes are intracellular and appear to reside in the inner plaque. They include the Bullous Pemphigoid Antigen 1 (BPAG1), which is the major target of the autoantibodies present in the blistering skin disease Bullous Pemphigoid (Sawamura et al., 1991; Tanaka et al., 1991), the HD-1 protein (Hieda et al., 1992), and the 6A5 antigen (Kurpakus et al., 1991). BPAG1 contains a region of homology with the desmosomal protein desmoplakin (Sawamura et al., 1991). Since the homology falls within a desmoplakin domain that directly binds to keratins (Stappenbeck and Green, 1992; Kouklis et al., 1994), it is likely that BPAG1 also interacts with the keratin filaments, thereby linking the innermost plaque of hemidesmomes to the cytoskeleton. In accordance with this hypothesis, the hemidesmosomes of BPAG1 knock-out mice lack the inner plate and are not connected to the keratin cytoskeleton (Guo et al., 1995). The molecular interactions between the transmembrane and intracellular components of hemidesmosomes are incompletely understood because the composition of the short filaments connecting the inner plaque to the juxta-membranous plaque is not known.

The expression of various deletion mutant forms of β_4 in hemidesmosome forming cells has revealed that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton is mediated by the β_4 cytoplasmic domain and, specifically, by a 303 amino acid region containing the first pair of type III Fn-like repeats and the Connecting Segment (Spinardi et al., 1993). The observation that the extracellular domain of $\alpha_6\beta_4$ binds to laminin 5 and the specialized cytoplasmic tail of β_4 subunit interacts with the hemidesmosomal cytoskeleton suggests that this integrin plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system. In accordance with this hypothesis, the introduction of a truncated tail-less β_4 subunit in cells possessing endogenous $\alpha_6\beta_4$ integrins and hemidesmosomes results in a dominant negative effect on hemidesmosome assembly (Spinardi et al., 1995). Since the tail-less integrin retains the ability to bind laminins, its dominant negative effect is likely to result from its ability to co-cluster with the endogenous wild-type receptor and block a conformational change or a signal necessary for hemidesmosome assembly. Interestingly, the cells which express the tail-less integrin do not show a defective interaction with laminins in short term adhesion assays, but they are significantly rounder and more detached from the culture substratum than control cells. This observation suggests that the main function of hemidesmosomes is to stabilize the $\alpha_6\beta_4$-mediated adhesion to the basement membrane.

Several observations indicate that the transmembrane connection between the basement membrane and the keratin filament system mediated by $\alpha_6\beta_4$ plays a crucial role in maintaining the integrity of skin and other stratified epithelia. Mutations in the β_3 and γ_2 laminin subunit genes (Aberdam et al., 1994; Pulkkinen et al., 1994a,b) and lack of expression of laminin 5 (Domloge-Hultsch et al., 1992) have been detected in the lethal form (Herlitz type) of Junctional Epidermolysis Bullosa (JEB), a blistering skin disease in which the hemidesmosomes are defective and the epidermis detaches from the basement membrane. Furthermore, mutations in the β_4 gene and greatly reduced or often absent expression of $\alpha_6\beta_4$ have been observed in a subset of JEB which is accompanied by pyloric atresia (Gil et al., 1994;Vidal et al., 1995). Finally, mice in which the β_4 gene has been knocked out display extensive detachment of the skin and other epithelial abnormalities (Arnoud Sonnenberg, personal communication). Since the β_4 knock-out mice die soon after birth, to examine the potential involvement of $\alpha_6\beta_4$ in Schwann cell myelination and T-cell differentiation it will be necessary to generate chimeric mice or to rescue the mutant mice from blistering by targeting the expression of a transgenic wild-type β_4 subunit to the skin. Although the above mentioned studies may have only partially uncovered the developmental roles of $\alpha_6\beta_4$, they have provided clear indication that the interaction of $\alpha_6\beta_4$ with laminin 5 at hemidesmosomes is of crucial physiological importance.

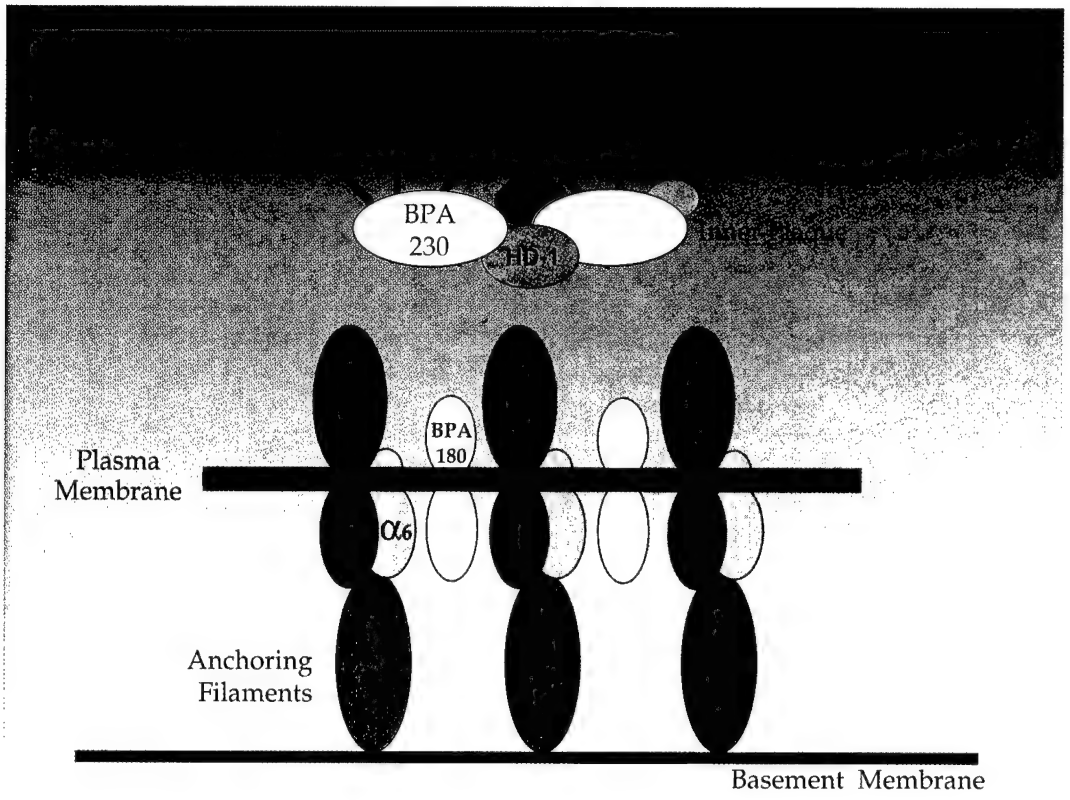


Fig. 1. Molecular architecture of the hemidesmosome.

INTRACELLULAR SIGNALING

The multifarious effects of laminins on cellular behavior, in particular their ability to influence proliferation and differentiation, suggest that laminin-binding results in intracellular signaling. What are the mechanisms by which the $\alpha_6\beta_4$ integrin transduces biochemical signals to the cell interior? The results of a recent series of experiments suggests that $\alpha_6\beta_4$ relies on its association with an intracellular tyrosine kinase for signal transduction (Mainiero et al., 1995). In this respect $\alpha_6\beta_4$ can be considered a binary tyrosine kinase receptor similar to many cytokine and immune receptors, which do not possess an intracellular catalytic domain but are physically and functionally associated with cytoplasmic tyrosine kinases (Kishimoto et al., 1994; Weiss and Littman, 1994). Laminin binding to $\alpha_6\beta_4$ causes activation of the associated kinase and consequently tyrosine phosphorylation of the β_4 subunit cytoplasmic domain. Since these events can be replicated by exposing the cells to beads coated with anti-$\alpha_6\beta_4$ antibodies, the mechanism by which ligand binding causes tyrosine phosphorylation of β_4 is likely to include dimerization or oligomerization of the integrin on the plasma membrane. This event is likely to bring the integrin-associated kinase in close proximity to its target sequences in the β_4 tail and to induce *trans* phosphorylation and activation of the kinase.

Immunoprecipitation experiments have indicated that the adaptor protein Shc forms a tight complex with the tyrosine phosphorylated β_4 subunit and is thereby phosphorylated on tyrosine residues, presumably by the integrin-associated kinase. The subsequent recruitment to the plasma membrane of the other adaptor protein Grb2, which exists in a complex with the *ras* GTP exchange factor mSOS, potentially links $\alpha_6\beta_4$ to the *ras* signaling pathways. Indeed, *ras* GTP-loading experiments have indicated that ligation of $\alpha_6\beta_4$ results in a significant activation of *ras* (F. Mainiero and F. G. Giancotti, unpublished results). The linkage of $\alpha_6\beta_4$ to *ras* and the various MAP kinase pathways directly or indirectly regulated by *ras* provides a mechanism by which laminins could regulate gene expression. Moreover, since the MAP kinase pathway activated by *ras* has been implicated in controlling proliferation or differentiation depending on the cellular context (Cowley et al., 1994), the signal transduction mechanism described above may explain the apparently paradoxical effects of laminins on morphogenesis and tumor progression.

In apparent contrast with the above results, it has been recently reported that overexpression of wild-type, but not tailless β_4 induces $p21^{WAF1/Cip1}$ expression and cell-cycle withdrawal in rectal carcinoma cells (Clarke et al., 1995). However, since this effect appears to occur independently of ligand binding to the integrin, it is possible that the unoccupied $\alpha_6\beta_4$ is somehow linked to the $p21^{WAF1/Cip1}$ pathway of growth suppression and that laminin binding reverses this connection and couples the integrin through Shc and Grb2 to *ras*. The observation that reversion of $\alpha_6\beta_4$-mediated adhesion causes dephosphorylation of an as yet unidentified 80 kDa protein (Xia et al., 1996) is also consistent with the hypothesis that ligated and unligated $\alpha_6\beta_4$ integrins impinge on distinct intracellular pathways.

As discussed above, the assembly of hemidesmosomes is likely to be triggered by the binding of laminin 5 to $\alpha_6\beta_4$ and to require the subsequent interaction of the β_4 cytoplasmic domain with cytoskeletal elements of hemidesmosomes. There is strong evidence indicating that $\alpha_6\beta_4$ signaling is an integral component of this process. The multiple β_4 sites phos-

phorylated in vivo by the $\alpha_6\beta_4$-associated kinase include a tyrosine activation motif (TAM) located in the Connecting Segment. The TAM is a bidentate phosphorylation motif which consists of two closely spaced tyrosine residues followed by a leucine at position + 3 and was originally identified in several immune receptors (Reth, 1989). Interestingly, $\alpha_6\beta_4$ is the first molecule outside the immune system with such a motif. Phenylalanine substitutions at the β_4 TAM disrupt the association of $\alpha_6\beta_4$ with hemidesmosomes, but do not affect recruitment of Shc and Grb2 (Mainiero et al., 1995). This result indicates that phosphorylation of the β_4 TAM is necessary for the assembly of hemidesmosomes, but not for the recruitment of Shc and Grb2. In accordance with the role of tyrosine phosphorylation in hemidesmosome assembly, inhibition of β_4 phosphorylation by the tyrosine kinase inhibitor herbimycin correlates with inhibition of hemidesmosomes (A. Pepe and F. G. Giancotti, unpublished results). Furthermore, the ability of tail-less β_4 to suppress hemidesmosome formation is reminiscent of the dominant negative effect that tail-less tyrosine kinase receptors exert on signal transduction and is consistent with the proposed role of $\alpha_6\beta_4$ signaling in hemidesmosome assembly (Spinardi et al., 1995).

The immune receptor TAMs provide a template for the binding of tyrosine kinases containing two tandem SH2 domains, such as *Syk* and ZAP70. Recruitment of these kinases to the TAMs is coupled with their activation and is crucial for subsequent downstream signaling events from immune receptors (Weiss and Littman, 1994). These observations suggest that the mechanism by which phosphorylation of the β_4 TAM regulates cytoskeletal assembly may involve binding of a protein containing two tandem SH2 domains. It is unlikely that this element is a cytoskeletal element which binds stably to $\alpha_6\beta_4$ because tyrosine phosphorylation of β_4 occurs only transiently after ligand binding. In fact, in stably adherent cells β_4 appears to be completely dephosphorylated, yet most of $\alpha_6\beta_4$ is in hemidesmosomes. In addition, phosphorylation of the TAM is unlikely to be sufficient for the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton because deletion mutagenesis experiments have indicated that the two type III fibronectin-like modules upstream of the TAM are also required for this process (L. Spinardi and F. G. Giancotti, unpublished results). The analogy with the immune receptors suggests that the β_4 TAM mediates a signal important for the assembly of hemidesmosomes by recruiting a protein kinase, phosphatase or adapter. The tyrosine kinases ZAP70 and *syk* appear to be restricted to the immune system and therefore their tissue distribution does not overlap with that of $\alpha_6\beta_4$. In addition, since the spacing between tyrosine 1422 and 1440 in β_4 is larger than the distance between the tyrosines in immune receptor TAMs, it is likely that the β_4 TAM has a distinct binding specificity and mediates the activation of a novel signal transduction pathway.

The model proposed above is illustrated in Fig. 2. Its major prediction is that the association of $\alpha_6\beta_4$ with the hemidesmososmal cytoskeleton and its linkage to *ras* are mediated by distinct divergent pathways. This suggests the possibility that the two pathways are differentially regulated in vivo, perhaps in response to different laminins or different oligomerization states of the same laminin matrix. Activation of each of the two pathways may also depend on the availability of the signaling components involved and therefore vary

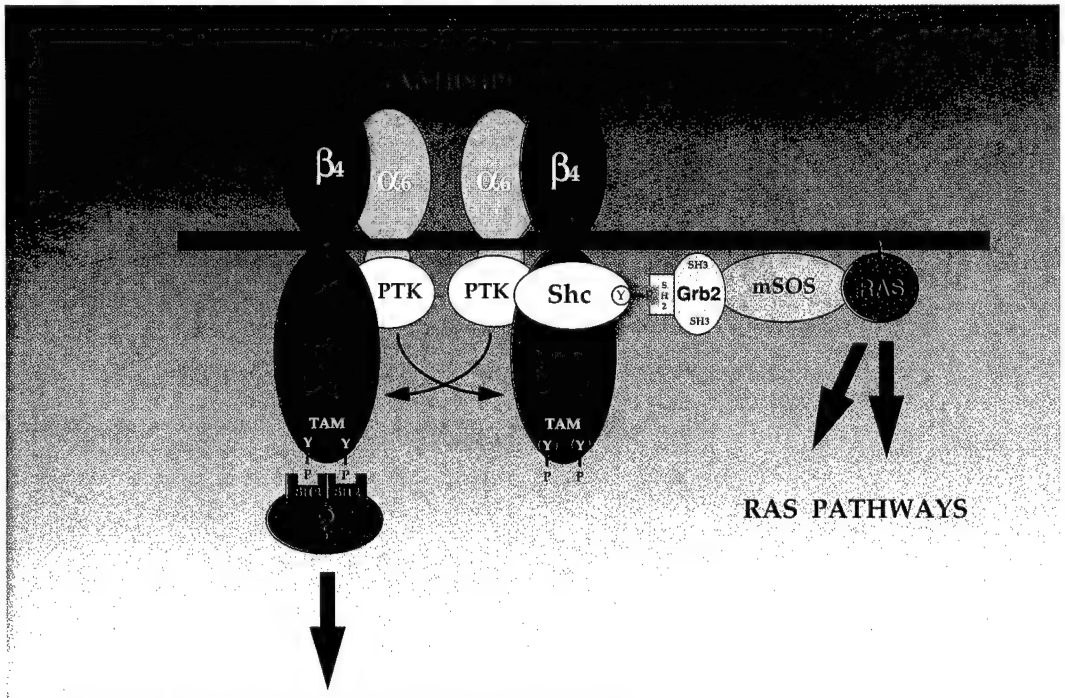


Fig. 2. Model of $\alpha_6\beta_4$-mediated signal transduction.

with cell type and developmental stage. These mechanisms would clearly provide flexibility to cellular responses to laminins.

CONCLUSION AND PERSPECTIVES

The above results delineate the basic mechanism by which the $\alpha_6\beta_4$ integrin transduces biochemical signals accross the plasma membrane and offer a number of avenues for future investigation. At the molecular level, it will be important to identify the tyrosine kinase associated with $\alpha_6\beta_4$ and define all the intracellular pathways activated by the integrin and their target genes. It is likely that $\alpha_6\beta_4$ activates the various MAP kinase pathways controlled by *ras*, but the extent to which each one is stimulated remains to be determined. In addition, the recruitment of Shc and Grb2 may link $\alpha_6\beta_4$ not only to *ras*, but also to other adaptor and effector molecules. A comprehensive understanding of the pathways activated by $\alpha_6\beta_4$ will be a first necessary step to elucidate the molecular basis by which the integrin can influence proliferation and differentiation.

Progression through the cell cycle and differentiation are usually mutually exclusive and require the activation of largely distinct sets of genes. It is intriguing that most growth and differentiation factors activate the *ras*-MAP kinase pathway and that MAP kinase can induce proliferation or differentiation depending on the cell type (Cowley et al., 1994). This observation suggests that MAP kinase may impinge on different transcription factors in different cell types or physiological situations. If this is the case, the final output of signaling through MAP kinase may depend on which set of target factors is available in any given situation. Alternatively, it is possible that the choice between growth and differentiation is controlled by specific pathways distinct from those activated by *ras*. Clearly, it will be important to resolve these issues with regard to signaling from both classical growth factor receptors and integrins.

The relationship between growth factor-dependent signaling and cell adhesion mediated by $\alpha_6\beta_4$ or other integrins needs further investigation. One cannot discount the exciting possibility that the final output of signaling from integrins may depend on the existence of as yet unidentified pathways impinging on genes distinct from those activated by growth and differentiation factors. Alternatively, as the above described studies on $\alpha_6\beta_4$ suggest, the pathways activated by soluble factors and extracellular matrix proteins may be largely overlapping. In both cases, it will be important to understand how the signals are integrated and what determines the quality of the final output.

Experiments of mutagenesis and dominant negative inhibition in cultured cells may help to understand the influence that signaling by $\alpha_6\beta_4$ may have on survival and proliferation. It is likely that the potential role of $\alpha_6\beta_4$ signaling in differentiation will be best addressed by molecular genetics studies in the mouse, especially if it will be possible to separate the adhesive and signaling function of the integrin. Finally, the pathway which controls the assembly of hemidesmosomes is likely to be specifically activated by $\alpha_6\beta_4$. To understand this pathway it will be necessary to identify the signaling component interacting with the phosphorylated β_4 TAM as well as its downtream targets. One possibility is that these targets include one or more cytoskeletal elements of hemidesmosomes. Proper modification of these molecules by the β_4 TAM signaling pathway may be necessary for their subsequent interaction with the β_4 cytoplasmic domain. Clearly, although studying $\alpha_6\beta_4$ has been particularly rewarding, much remains to be learned.

The author is the recipient of awards from the Lucille P. Markey Charitable Trust and the Irma T. Hirschl Trust. Work in his laboratory is supported by grants from the NIH and the USAMRMC.

REFERENCES

Aberdam, D., Galliano, M.-F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein, E. H., Ortonne, J.-P. and Meneguzzi, G. (1994). Herlitz's junctional epidermolysis bullosa is linked to mutations in the gene (LAMC2) for the γ2 subunit of nicein/kalinin (laminin 5). *Nature Genet.* **6**, 299-304.

Bergstraesser, L. M., Srinivasan, G., Jones, J. C. R., Stahl, S. amd Weitzman, S. A. (1995). Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. *Am. J. Pathol.* **147**, 1823-1839.

Bunge, R. P., Bunge, M. B. and Eldridge, C. F. (1986). Linkage between axonal ensheathment and basal lamina production by Schwann cells. *Annu. Rev. Neurosci.* **9**, 305-328.

Carey, D. J., Todd, M. S. and Rafferty, C. M. (1986). Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. *J. Cell Biol.* **102**, 2254-2263.

Carico, E., French, D., Bucci, B., Falcioni, R., Vecchione, A. and Mariani-Costantini, R. (1993). Integrin β_4 expression in the neoplastic progression of cervical epithelium. *Gynecol. Oncol.* **49**, 61-66.

Carter, W. G., Kaur, P., Gil, S. G., Gahr, P. J. and Wayner, E. A. (1990a). Distinct functions for integrins $\alpha_3\beta_1$ in focal adhesions and $\alpha_6\beta_4$/Bullous Pemphigoid Antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* **111**, 3141-3154.

Carter, W. G., Wayner, E. A., Bouchard, T. S. and Kaur, P. (1990b). The role of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in cell-cell and cell-substrate adhesion of human epidermal cells. *J. Cell Biol.* **110**, 1387-1404.

Carter, W. G., Ryan, M. C. and Gahr, P. J. (1991). Epiligrin, a new cell adhesion ligand for integrin $\alpha_3\beta_1$ in epithelial basement membranes. *Cell* **65**, 599-610.

Chang, A. C., Wadswarth, S. and Coligan, J. E. (1993). Expression of merosin in the thymus and its interaction with thymocytes. *J. Immunol.* **151**, 1789-1801.

Clark, E. A. and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-239.

Clarke, A. S., Lotz, M. M., Chao, C. and Mercurio, A. M. (1995). Activation of the p21 pathway of growth arrest and apoptosis by the β_4 integrin cytoplasmic domain. *J. Biol. Chem.* **270**, 22673-22676.

Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841-852.

Dedhar, S., Saulnier, R., Nagle, R. and Overall, C. M. (1993). Specific alterations in the expression of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins in highly invasive and metastatic variants of human prostate carcinoma cells selected by in vitro invasion through reconstituted basement membrane. *Clin. Exp. Metastasis* **11**, 391-400.

Domloge-Hultsch, N., Gammon, W. R., Briggaman, R. A., Gil, S. G., Carter, W. G. and Yancey, K. B. (1992). Epiligrin, the major human keratinocyte integrin ligand is a target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. *J. Clin. Invest.* **90**, 1628-1633.

Einheber, S., Milner, T. A., Giancotti, F. G. and Salzer, J. L. (1993). Axonal regulation of Schwann cell integrin expression suggests a role for $\alpha_6\beta_4$ in myelination. *J. Cell Biol.* **123**, 1223-1236.

Ellison, J. and Garrod, D. R. (1984). Anchoring filaments of the amphibian epidermal-dermal junction traverse the basal lamina entirely from the plasma membrane of hemidesmosomes to the dermis. *J. Cell Sci.* **72**, 163-172.

Enenstein, J. and Kramer, R. H. (1994). Confocal microscopic analysis of integrin expression on the microvasculature and its sprouts in the neonatal foreskin. *J. Invest. Dermatol.* **103**, 381-386.

Engvall, E. (1993). Laminin variants: why, where and when? *Kidney Int.* **43**, 2-6.

Feltri, M. L., Scherer, S. S., Nemni, R., Kamholz, J., Vogelbacker, H., Oronzi Scott, M., Canal, N., Quaranta, V. and Wrabetz, L. (1994). β_4 integrin expression in myelinating Schwann cells is polarized, developmentally regulated and axonally dependent. *Development* **120**, 1287-1301.

Giancotti, F. G., Stepp, M. A., Suzuki, S., Engvall, E. and Ruoslahti, E. (1992). Proteolytic processing of endogenous and recombinant β_4 integrin subunit. *J. Cell Biol.* **118**, 951-959.

Giancotti, F. G. and Mainiero, F. (1994). Integrin-mediated adhesion and signaling in tumorigenesis. *BBA Rev. Cancer* **1198**, 47-64.

Gil, S. G., Brown, T. A., Ryan, M. C. and Carter, W. G. (1994). Junctional epidermolysis bullosis: defects in expression of epiligrin/nicein/kalinin and integrin β_4 that inhibit hemidesmosome formation. *J. Invest. Dermatol.* **103**, 31S-38S.

Giudice, G. J., Emery, D. J. and Diaz, L. A. (1992). Cloning and primary structural analysis of the Bullous Pemphigoid Autoantigen BP180. *J. Invest. Dermatol.* **99**, 243-250.

Green, H. (1977). Terminal differentiation of cultured human epidermal cells. *Cell* **11**, 405-416.

Guo, L., Degenstein, L., Dowling, J., Yu, Q.-C., Wollmann, R., Perman, B. and Fuchs, E. (1995). Gene targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurological degeneration. *Cell* **81**, 233-243.

Hall, P. A. and Watt, F. M. (1989). Stem cells: the generation and maintenance of cellular diversity. *Development* **106**, 619-633.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**, 245-254.

Hieda, Y., Nishizawa, Y., Uematsu, J. and Owaribe, K. (1992). Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* **116**, 1497-1506.

Hogervorst, F., Kuikman, I., von dem Borne, A. E. G. Kr. and Sonnenberg, A. (1990). Cloning and sequence analysis of β_4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J.* **9**, 745-770.

Hopkinson, S. B., Baker, S. E. and Jones, J. C. R. (1995). Molecular genetic studies of a human epidermal autoantigen (the 180-kD Bullous Pemphigoid Antigen/BP180): identification of functionally important sequences within the BP180 molecule and evidence for an interaction between BP180 and α_6 integrin. *J. Cell Biol.* **130**, 117-125.

Jaspars, L. H., van der Linden, H. C., Scheffer, G. L., Scheper, R. J. and Meijer, C. J. L. M. (1993). Monoclonal antibody 4C7 recognizes an endothelial basement membrane component that is selectively expressed in capillaries of lymphoid follicles. *J. Pathol.* **170**, 121-128.

Jerdan, J. A., Michels, R. G. and Glaser, B. M. (1991). Extracellular matrix of newly forming vessels: an immunohistochemical study. *Microvasc. Res.* **42**, 255-265.

Jones, J., Sugiyama, M., Watt, F. M. and Speight, P. M. (1993). Integrin expression in normal, hyperplastic, dysplastic, and malignant oral epithelium. *J. Pathol.* **169**, 235-243.

Kajiji, S., Tamura, R. N. and Quaranta, V. (1989). A novel integrin ($\alpha_E\beta_4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.* **8**, 673-680.

Kennel, S. J., Godfrey, V., Chang, L. Y., Lankfork, T. K., Foote, L. J. and Makkinje, A. (1992). The integrin β_4 subunit is displayed on a restricted subset of endothelium in mice. *J. Cell Sci.* **101**, 145-150.

Kimmel, K. A. and Carey, T. E. (1986). Altered expression in squamuos carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.* **46**, 3614-2623.

Kishimoto, T., Taga, T. and Akira, S. (1994). Cytokine signal transduction. *Cell* **76**, 253-262.

Klein, S., Giancotti, F. G., Presta, M., Albelda, S. M., Buck, C. A. and Rifkin, D. B. (1993). Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell* **4**, 973-982.

Knox, J. D., Cress, A. E., Clark, V., Menriquez, L., Affinito, K.-S., Dalkin, B. L. and Nagle, R. B. (1994). Differential expression of extracellular matrix molecules and α_6-integrins in the normal and neoplastic prostate. *Am. J. Pathol.* **145**, 167-174.

Kouklis, P., Hutton, E. and Fuchs, E. (1994). Making the connection: keratin intermediate filaments and desmosomes. *J. Cell Biol.* **127**, 1049-1060.

Koukoulis, G. K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V. and Gould, V. E. (1991). Immunohistochemical localization of integrins in the normal, hyperplastic and neoplastic breast. *Am. J. Pathol.* **139**, 787-799.

Kurpakus, M. A. and Jones, J. C. R. (1991). A novel hemidesmosomal plaque component: tissue distribution and incorporation into assembling hemidesmosomes in an in vitro model. *Exp. Cell Res.* **194**, 139-146.

Lee, E. C., Lotz, M. M., Steele, G. D. and Mercurio, A. M. (1992). The integrin $\alpha_6\beta_4$ is a laminin receptor. *J. Cell Biol.* **117**, 671-678.

Legan, P. K., Collins, J. E. and Garrod, D. R. (1992). The molecular biology of desmosomes and hemidesmosomes: 'what's in a name?' *BioEssays* **14**, 385-393.

Magner, W. J., Chang, A. C., Owens, J., Hong, M.-J. P., Brooks, A. and Coligan, J. E. (1995). Aberrant differentiation of thymocytes in mice lacking laminin-2. *Mol. Biol. Cell* **6**, 280a.

Mainiero, F., Pepe, A., Wary, K. K., Spinardi, L., Mohammadi, M., Schlessinger, J. and Giancotti, F. G. (1995). Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO J.* **14**, 4470-4481.

Mariani-Costantini, R., Falcioni, R., Battista, P., Zupi, G., Kennel, S. J., Colasante, A., Venturo, I., Gallo-Curcio, C. and Sacchi, A. (1990). Integrin (α_6/β_4) expression in human lung cancer as monitored by specific monoclonal antibodies. *Cancer Res.* **50**, 6107-6112.

Marinkovich, M. P., Lunstrum, G. P., Keene, D. R. and Burgeson, R. E. (1992). The dermal-epidermal junction of human skin contains a novel laminin variant. *J. Cell Biol.* **119**, 695-703.

Menon, G. K., Grayson, S. and Elias, P. M. (1985). Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. *J. Invest. Dermatol.* **84**, 508-512.

Mercurio, A. M. (1995). Laminin receptors: achieving specificity through cooperation. *Trends Cell Biol.* **5**, 419-423.

Natali, P. G., Nicotra, M. R., Botti, C., Mottolese, M., Bigotti, A. and Segatto, O. (1992). Changes in expression of α_6/β_4 integrin heterodimer in primary and metastatic breast cancer. *Brit. J. Cancer* **66**, 318-322.

Niessen, C. M., Hogervorst, F., Jaspars, L. H., De Melker, A. A., Delwel, G. O., Hulsman, E. H. M. S., Kuikman, I. and Sonnenberg, A. (1994). The $\alpha_6\beta_4$ integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.* **2111**, 360-367.

Paulsson, M. (1992). Basement membrane proteins: structure, assembly, and cellular interactions. *Crit. Rev. Biochem. Mol. Biol.* **27**, 93-127.

Pyke, C., Romer, J., Kallunki, P., Lund, L. R., Ralfkiaer, E., Dano, K. and Triggvason, K. (1994). The γ 2 chain of kalinin/laminin-5 is preferentially expressed in invading malignant cells in human cancers. *Am. J. Pathol.* **145**, 782-791.

Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K. and Triggvason, K. (1995). Laminin 5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. *Cancer Res.* **55**, 4132-4139.

Pulkkinen, L., Christiano, A. M., Airenne, T., Haakana, H., Tryggvason, K. and Uitto, J. (1994a). Mutations in the γ 2 chain gene (LAMC2) of kalinin/laminin 5 in the junctional forms of epidermolysis bullosa. *Nature Genet.* **6**, 293-298.

Pulkkinen, L., Christiano, A. M., Gerecke, G., Wolfe Wagman, D., Burgeson, R. E., Pittelkow, M. R. and Uitto, J. (1994b). A homozygous nonsense mutation in the β 3 chain gene of laminin 5 (LAMB3) in Herlitz junctional epidermolysis bullosa. *Nature Genet.* **6**, 293-298.

Reth, M. (1989). Antigen receptor tail clue. *Nature* **338**, 383-384.

Rousselle, P., Lunstrum, G. P., Keene, D. R. and Burgeson, R. E. (1991). Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.* **114**, 567-576.

Sanes, J. R., Engvall, E., Butkowski, R. and Hunter, D. D. (1990). Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. *J. Cell Biol.* **111**, 1685-1699.

Savoia, P., Trusolino, L., Pepino, E., Cremona, O. and Marchisio, P. C. (1993). Expression and topography of integrins and basement membrane proteins in epidermal carcinomas. *J. Invest. Dermatol.* **101**, 352-358.

Sawamura, D., Li, K., Chu, M. L. and Uitto, J. (1991). Human Bullous Pemphigoid Antigen (BPAG1): amino acid sequences deduced from cloned cDNAs predict biologically important peptide segments and protein domains. *J. Biol. Chem.* **266**, 17784-17790.

Schenk, P. (1979). The fate of hemidesmosomes in laryngeal carcinoma. *Arch. Oto-Rhino-Laringol.* **222**, 187-198.

Schwarz, M. A., Owaribe, K., Kartenbeck, J. and Franke, W. W. (1990). Desmosomes and hemidesmosomes: constitutive molecular components. *Annu. Rev. Cell Biol.* **6**, 461-491

Schwarz, M. A., Shaller, M. D. and Ginsberg, M. H. (1995). Integrins:

emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* **11**, 549-599.

Shienvold, F. L. and Kelly, D. E. (1976). The hemidesmosome: new fine structural features revealed by freeze-fracture techniques. *Cell Tissue Res.* **172**, 289-307.

Sonnenberg, A., Linders, C. J. T., Daams, J. H. and Kennel, S. J. (1990). The $\alpha_6\beta_1$ (VLA-6) and $\alpha_6\beta_4$ protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.* **96**, 207-217.

Spinardi, L., Ren, Y.-L., Sanders, R. and Giancotti, F. G. (1993). The β_4 subunit cytoplasmic domain mediates the interaction of $\alpha_6\beta_4$ integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell* **4**, 871-884.

Spinardi, L., Einheber, S., Cullen, T., Milner, T. A. and Giancotti, F. G. (1995). A recombinant tail-less integrin $\alpha_6\beta_4$ subunit disrupts hemidesmosomes, but does not suppress $\alpha_6\beta_4$-mediated cell adhesion to laminins. *J. Cell Biol.* **129**, 473-487.

Stappenbeck, T. S. and Green, K. J. (1992). The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* **116**, 1197-1209.

Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J. and Gipson, I. K. (1990). Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Nat. Acad. Sci. USA* **87**, 8970-8974.

Sunada, Y., Bernier, S. M., Kozak, C. A., Yamada, Y. and Campbell, K. P. (1994). Deficiency of merosin in dystrophic *dy* mice and genetic linkage of laminin M chain gene to *dy* locus. *J. Biol. Chem.* **269**, 13729-13732.

Suzuki, S. and Naitoh, Y. (1990). Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.* **9**, 757-763.

Tanaka, T., Parry, D. A. D., Kovtun, V. K., Steiner, P. M. and Stanley, J. R. (1991). Comparison of molecularly cloned Bullous Pemphigoid Antigen to Desmoplakin 1 confirms that they define a new family of cell adhesion junction plaque proteins. *J. Biol. Chem.* **266**, 12555-12559.

Tennenbaum, T., Yuspa, S. H., Grover, A., Castronovo, V., Sobel, M. E., Yamada, Y. and De Luca, L. M. (1992). Extracellular matrix receptors and mouse skin carcinogenesis: altered expression linked to appearance of early markers of tumor progression. *Cancer Res.* **52**, 2966-2976.

Tennenbaum, T., Weiner, A. K., Belanger, A. J., Glick, A. B., Hennings, H. and Yuspa, S. H. (1993). The suprabasal expression of $\alpha_6\beta_4$ integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. *Cancer Res.* **53**, 4803-4810.

Tennenbaum, T., Belanger, A. J., Glick, A. B., Tamura, R., Quaranta, V. and Yuspa, S. H. (1995). A splice variant of α_6 integrin is associated with malignant conversion in mouse skin tumorigenesis. *Proc. Nat. Acad. Sci. USA* **92**, 7041-7045.

Timpl, R. (1989). Structure and biological activities of basement membrane proteins. *Eur. J. Biochem.* **180**, 487-502.

Vidal, F., Aberdam, D., Miquell, C., Christiano, A. M., Pulkkinen, L., Uitto, J., Ortonne, J.-P. and Meneguzzi, G. (1995). Integrin β_4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nature Genet.* **10**, 229-234.

Wadsworth, S., Halvorson, M. J. and Coligan, J. E. (1992). Developmentally regulated expression of the β_4 integrin on immature mouse thymocytes. *J. Immunol.* **149**, 421-428.

Weiss, A. and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263-274.

Wolf, G. T., Carey, T. E., Schmaltz, S. P., McClatchey, K. D., Poore, J., Glaser, L., Hayashida, D. J. S. and Hsu, S. (1990). Altered antigen expression predicts outcome in squamous cell carcinomas of the head and neck. *J. Nat. Cancer Inst.* **82**, 1566-1572.

Xia, Y., Gil, S. G. and Carter, W. G. (1996). Anchorage mediated by integrin $\alpha_6\beta_4$ to laminin 5 (epiligrin) regulates tyrosine phosphorylation of a membrane associated 80 kD protein. *J. Cell Biol.* **132**, 727-740.

Xu, H., Christmas, P., Wu, X.-R., Wewer, U. M. and Engvall, E. (1994). Defective muscle basement membrane and lack of M-laminin in the dystrophic *dy/dy* mice. *Proc. Nat. Acad. Sci. USA* **91**, 5572-5576.

SUBMISSION OF MANUSCRIPTS

Journal of Cell Science publishes critical work over the full range of cell biology. Papers are called for that deal with all aspects of both prokaryotic and eukaryotic cells, the single most important criterion for acceptance being scientific excellence. Short surveys of topical subjects appear in each issue under the general title *Commentary*.

Length of manuscript

Manuscripts including all text, references and figure legends should not exceed 8,000 words plus 4 page equivalents of figures and tables. Manuscripts longer than this will only be accepted at the editors' discretion.

Where to send

Manuscripts should be sent to the most appropriate editor (see list of addresses opposite). If you need advice, please contact the Editorial Office, or Editor-in-Chief.

Please supply your address, telephone and fax numbers for all correspondence about your manuscript.

For Customs purposes, please mark all packages as 'No Commercial Value', otherwise charges are levied.

What to send

Please send 3 copies of the manuscript and figures with the completed 'Preparation of manuscripts' check list. Copies of the figures must be of suitable standard for reviewers to judge the quality of the work (photocopies of photographs are not acceptable). The original illustrations and electronic files should *not* be included initially. These should be sent with your revised manuscript, when requested, i.e. after editor's and referees' comments have been incorporated.

Proofs

Authors will receive one set of proofs, which should be corrected and returned to the Editorial Office (a photocopy should be made and retained by the author). No alteration should be made to proofs other than those needed to correct printer's errors. All corrections, including those to figures, must be marked on the proof.

Reprints

Authors will receive 50 reprints free of charge.

Colour plates

These can be reproduced free of charge at the discretion of the editor. Any charge for excessive use of colour will also be at the editor's discretion.

Journal of Cell Science Editorial Office

Production Editor:

Dr Dawn E. Walters

Tel: (0)1223 424430

Fax: (0)1223 423353

E-mail: cob@cambridge.cityscape.co.uk

Editors

Contents

Volume 109 (6) 1996

The Company of Biologists Limited, Bidder Building, 140 Cowley Road, Cambridge CB4 4DL, UK
ISSN: 0021-9533

The Intracellular Functions of $\alpha_6\beta_4$ Integrin Are Regulated by EGF

Fabrizio Mainiero, Angela Pepe, Mitchell Yeon, Yunling Ren, and Filippo G. Giancotti

Department of Pathology, Kaplan Cancer Center, New York University School of Medicine, New York 10016

Abstract. Upon ligand binding, the $\alpha_6\beta_4$ integrin becomes phosphorylated on tyrosine residues and combines sequentially with the adaptor molecules Shc and Grb2, linking to the *ras* pathway, and with cytoskeletal elements of hemidesmosomes. Since $\alpha_6\beta_4$ is expressed in a variety of tissues regulated by the EGF receptor (EGFR), we have examined the effects of EGF on the cytoskeletal and signaling functions of $\alpha_6\beta_4$. Experiments of immunoblotting with anti-phosphotyrosine antibodies and immunoprecipitation followed by phosphoamino acid analysis and phosphopeptide mapping showed that activation of the EGFR causes phosphorylation of the β_4 subunit at multiple tyrosine residues, and this event requires ligation of the integrin by laminins or specific antibodies. Immunoprecipitation experiments indicated that stimulation with EGF does not result in association of $\alpha_6\beta_4$ with Shc. In contrast, EGF can partially suppress the recruitment of Shc to ligated $\alpha_6\beta_4$. Immunofluorescent analysis revealed that EGF treatment does not induce increased assembly of hemidesmosomes, but instead causes a deterioration of these adhesive structures. Finally, Boyden chamber assays indicated that exposure to EGF results in upregulation of $\alpha_6\beta_4$-mediated cell migration toward laminins. We conclude that EGF-dependent signals suppress the association of activated $\alpha_6\beta_4$ with both signaling and cytoskeletal molecules, but upregulate $\alpha_6\beta_4$-dependent cell migration. The changes in $\alpha_6\beta_4$ function induced by EGF may play a role during wound healing and tumorigenesis.

To fully understand embryonic development, tissue repair, and tumor invasion, it is important to elucidate the mechanisms by which growth factor– and integrin-dependent signals are integrated inside cells. It is known that integrins transmit positional cues from the extracellular matrix to the cell interior, and the mechanisms by which these signals affect cellular responses to growth and differentiation factors are being actively investigated (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994; Schwartz et al., 1995). Conversely, growth factors and cytokines can modulate a number of integrin-dependent functions, including cell adhesion (Serve et al., 1995; Kinashi et al., 1995), cell migration (Chen et al., 1993; Matthay et al., 1993; Klemke et al., 1994), and cytoskeletal organization (Ridley and Hall, 1992; Ridley et al., 1992), but the mechanisms underlying these phenomena are less clear.

The interaction between growth factor receptors and integrins has been largely examined in fibroblasts and platelets. Most of the studies have focused on the focal adhesion kinase $p125^{FAK}$ (Schaller et al., 1992). In addition to being activated and undergoing autophosphorylation in response to ligation of β_1 and β_3 integrins (Guan and Shalloway, 1992; Hanks et al., 1992; Lipfert et al., 1992), $p125^{FAK}$ is the target of signals originating from a number of growth factors and mitogenic neuropeptides (Zachary and Rozengurt, 1992). The activation of $p125^{FAK}$ has been linked to changes potentially important for the regulation of actin cytoskeleton, such as the phosphorylation of paxillin and tensin (Burridge et al., 1992; Bockholt and Burridge, 1993) and the activation of Rho (McNamee et al., 1992; Chong et al., 1994) and PI-3 kinase (Chen and Guan, 1994). In addition, activated $p125^{FAK}$ can combine with the Grb2/mSOS complex potentially leading to stimulation of the *ras*-MAP (mitogen-activated protein) kinase pathway (Schlaepfer et al., 1994), and insulin stimulation promotes association of the $\alpha_v\beta_3$ integrin with the Insulin Receptor Substrate 1 and the Grb2/mSOS complex (Vuori and Ruoslahti, 1994). These observations suggest that integrin- and growth factor–dependent signals may converge on $p125^{FAK}$ and Insulin Receptor Substrate 1 to regulate gene expression and the actin cytoskeleton.

Much less is known about the integration of growth factor– and integrin-dependent signals in epithelial and other cells that are in contact with the basement membrane. The $\alpha_6\beta_4$ integrin is expressed in epithelial, endothelial, and Schwann cells and binds to various isoforms of the basement membrane component laminin (Lee et al., 1992; Niessen et al., 1994; Spinardi et al., 1995). Our previous studies have focused on the mechanisms by which this integrin interacts with the cytoskeleton and with signaling molecules. In contrast to other integrins that localize to fo-

Address all correspondence to Filippo G. Giancotti, Department of Pathology, MSB 548, New York University School of Medicine, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-5343. Fax: (212) 263-8211. e-mail: giancF01@mcrcr6.med.nyu.edu.

Y. Ren's present address is 21-17 36th Street, Astoria, NY 11105.

cal adhesions or otherwise interact with the actin filament system, $\alpha_6\beta_4$ is found in hemidesmosomes in close proximity to molecules linking to the keratin filament system (Carter et al., 1990; Stepp et al., 1990). There is evidence indicating that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton requires the uniquely large cytoplasmic domain of β_4 and specifically a ~300–amino acid region, which includes the first two type III fibronectin–like modules and the connecting segment (Spinardi et al., 1993). The ability of a tail-less mutant β_4 subunit to produce a dominant negative effect on the assembly of hemidesmosomes without suppressing cell adhesion to laminins indicates that $\alpha_6\beta_4$ plays an essential role in organizing the hemidesmosomal cytoskeleton (Spinardi et al., 1995). Taken together, these observations suggest that laminin binding to $\alpha_6\beta_4$ promotes the nucleation of hemidesmosomal cytoskeleton, and this activity is mediated by the β_4 cytoplasmic domain.

Recent studies have indicated that ligation of the extracellular portion of $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit, and this event is mediated by protein kinase(s) physically associated with the integrin. Coimmunoprecipitation experiments have shown that, upon ligation of the extracellular portion of $\alpha_6\beta_4$, the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β_4 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor protein Grb2, thereby potentially linking $\alpha_6\beta_4$ to the *ras* pathway. The β_4 subunit is phosphorylated on multiple tyrosine residues *in vivo*, including a tyrosine-based activation motif (TAM)[1] resembling those found in the T cell and B cell receptors. Since phenylalanine substitutions at the β_4 TAM disrupt the association of $\alpha_6\beta_4$ with hemidesmosomes, but do not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2, distinct sites in $\alpha_6\beta_4$ mediate assembly of the hemidesmosomal cytoskeleton and linkage to the *ras* pathway (Mainiero et al., 1995).

The $\alpha_6\beta_4$ integrin is expressed in a variety of epithelial tissues that are regulated by the EGF (Sonnenberg et al., 1990). In this study, we have examined the effects of EGF on the cytoskeletal and signaling functions of $\alpha_6\beta_4$. Our results indicate that activation of the EGF receptor (EGFR) causes tyrosine phosphorylation of the β_4 subunit, but this event is not followed by association of the integrin with Shc or by increased assembly of hemidesmosomes. In contrast, EGF-dependent signals interfere with the ability of activated $\alpha_6\beta_4$ to associate with both signaling and cytoskeletal molecules. Exposure to EGF causes deterioration of hemidesmosomes and leads to increased $\alpha_6\beta_4$-mediated cell migration toward laminins.

Materials and Methods

Cell Lines, Transfections, Antibodies, and Extracellular Matrix Molecules

Human epidermoid carcinoma A431 cells were cultured in DME with 5% bovine FCS. Mouse mammary RAC-11P/SD cells (Sonnenberg et al., 1993) and rat bladder 804G cells (Izumi et al., 1981) were cultured in DME with 10% FCS or bovine calf serum, respectively. Human primary keratinocytes were cultured in keratinocyte growth medium (GIBCO BRL, Gaithersburg, MD). The 804G cells were cotransfected with the expression vector pRK5-hEGF-R, encoding a full-length human EGFR (Ullrich et al., 1984), and the hygromycin resistance plasmid pHBO by the calcium coprecipitation method (Giancotti et al., 1994). Stable cell lines expressing moderate levels of recombinant EGFR (25–35 times lower than the endogenous EGFR in A431 cells) were selected by fluorescence activated cell sorting analysis and cultured with medium supplemented with 200 μg/ml hygromycin (Calbiochem-Novabiochem Corp., La Jolla, CA). NIH-3T3 cells overexpressing a recombinant human EGFR (clone HER 14) (Honegger et al., 1987) were cultured in DME supplemented with 10% bovine calf serum and geneticin (GIBCO BRL).

The mAb 3E1 reacting with the extracellular portion of human β_4 and the rabbit polyclonal antiserum to the COOH-terminal peptide of β_4 were described previously (Giancotti et al., 1992). The mAbs BV7 and TS2/16 bind to the extracellular portion of the human β_1 subunit (Martin-Padura et al., 1994; Arroyo et al., 1992). The rabbit antiserum to the cytoplasmic domain of α_v was previously described (Vogel et al., 1993). The anti–major histocompatibility complex (MHC) mAb W6.32 reacts with human and cultured rat cells (Kahn-Perles et al., 1987). The rabbit polyclonal anti-P-Tyr serum #72 was produced according to published procedures (Kamps and Sefton, 1988). The monoclonal anti-P-Tyr antibody 4G10 was obtained from UBI (Lake Placid, NY). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum #554 was obtained by immunizing a rabbit with a glutathione-*S*-transferase fusion protein comprising the SH2 domain of the protein. The bullous Pemphigoid antigen 2 (BPAG 2)–specific rabbit polyclonal antiserum was raised by immunization with a glutathione-*S*-transferase fusion protein comprising the major antigenic determinant of the mouse protein in the laboratory of Jouni Uitto (Thomas Jefferson University, Philadelphia, PA). The antiserum against the $\alpha_5\beta_1$ integrin purified from human placenta was generated in the laboratory of Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) as previously described (Argraves et al., 1986). This antiserum cross-reacts with rodent β_1 integrins and blocks their function. The agarose-coupled 1G2 mAb was purchased from Oncogene Science (Uniondale, NY).

Human plasma fibronectin and human placental laminin 4 were purchased from GIBCO BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg et al., 1993; Spinardi et al., 1995).

Biochemical Methods

To test the effect of EGF on $\alpha_6\beta_4$, subconfluent A431 cells were serum starved and then treated with human recombinant EGF (Intergen Co., Purchase, NY). When indicated, the cells were detached by 10 mM EDTA and either kept in suspension or plated on dishes coated with extracellular matrix proteins before EGF stimulation. To examine the effect of selective ligation of $\alpha_6\beta_4$, the cells were plated on fibronectin-coated dishes, and then incubated with sulfate polystyrene latex beads coated with the 3E1 or control mAbs TS2/16 and W6.32. Stimulation of suspended cells with antibody-coated beads was performed as previously described (Mainiero et al., 1995). At the end of incubation, the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycolate, 0.1% sodium dodecyl sulfate) or lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM EDTA, and 1 mM EGTA (all from Sigma Chemical Co., St. Louis, MO).

Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti et al., 1992). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

Phosphopeptide mapping was performed essentially as described by Boyle et al. (1991). Serum-starved cells were labeled metabolically with [^{32}P]orthophosphate (3 mCi/ml; ICN Biochemicals, Inc., Irvine, CA) for 3 h and then either treated with 250 ng/ml EGF for 5 min at 37°C or with 500 μM sodium orthovanadate and 3 mM H_2O_2 for 10 min at 37°C. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β_4 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma Chemical Co.), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μl of 50 mM phosphate buffer, pH 7.8, containing 25 μg

1. *Abbreviations used in this paper*: BPAG 2, bullous pemphigoid antigen 2; EGFR, EGF receptor; MHC, major histocompatibility complex; TAM, tyrosine-based activation motif.

of *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid) (1.5 kV, 50 min) and in the second, by ascending chromatography in phospho chromatography buffer (37.5% *n*-butanol, 25% pyridin, 7.5% acetic acid).

Phosphoamino acid analysis was performed as described by Boyle et al. (1991). ^{32}P-labeled β_4 was eluted from fixed polyacrylamide gels and precipitated with 20% TCA. ^{32}P-labeled peptides were scraped off TLC plates, eluted in 20% acetonitrile and 0.08% trifluoroacetic acid, and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC: electrophoresis in pH 1.9 buffer for the first dimension (1.5 kV, 40 min) and in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension (1.5 kV, 30 min). Nonradioactive standards were detected by ninhydrin staining.

Adhesion and Migration Assays

Adhesion assays were performed essentially as previously described (Giancotti et al., 1985). Before the assay, the cells were serum starved and either treated with 100 ng/ml EGF for 5 min or left untreated. After detachment by incubation in 10 mM EDTA, they were washed and plated on extracellular matrix–coated plates in the presence of anti-β_1 serum at 1:50. The results were quantitated as previously described (Giancotti et al., 1986).

Cell migration assays were performed by using modified Boyden chambers containing porous (8-μm) polycarbonate membranes (Nunc, Roskilde, Denmark). To measure migration toward fibronectin and laminin 4, the lower aspect of the membrane was coated with 10 μg/ml of each extracellular matrix protein. To measure migration toward laminin 5, RAC-11P/SD cells were cultured on the lower aspect of the filter, and their laminin 5–containing matrix was prepared as described previously (Sonnenberg et al., 1993; Spinardi et al., 1995). Cells (50,000) were added to the upper chamber in 200 μl of serum-free DME supplemented with 1% ITS+ (Collaborative Research, New Bedford, MA). EGF (50 ng/ml) and PDGF (5 ng/ml) were placed in the lower migration chamber in 500 μl of the same medium. When indicated, the inhibitory anti-β_1 serum was added at 1:50 final dilution. After 12 or 48 h of incubation at 37°C, the cells that had migrated across the membrane were fixed with 3% paraformaldehyde, stained with crystal violet, and counted.

Immunofluorescence

The 804G transfectants and primary human keratinocytes were cultured on glass coverslips, starved for ~24 h and then treated with EGF, PDGF, or left untreated. After extraction with PBS containing 0.2% Triton X-100 for 5 min on ice, the cells were fixed with methanol and stained for 45 min with the various antibodies. The anti-β_4 cytoplasmic peptide rabbit serum was diluted 1:200. The anti-BPAG 2 IgGs were used at 25 μg/ml, and the 3E1 mAb was used at 5 μg/ml. After extensive washing, the cells were incubated for 45 min with 0.5–1 μg/ml affinity-purified FITC-conjugated goat anti–rabbit or anti–mouse IgGs (Molecular Probes, Inc., Eugene, OR). The coverslips were mounted in Citi-Fluor (Chemical Laboratory of the University of Kent, Canterbury, UK). Samples were examined with a fluorescent microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY).

Results

EGF-mediated Tyrosine Phosphorylation of the β_4 Subunit

To test the hypothesis of a potential link between the intracellular responses elicited by EGF and the function of $\alpha_6\beta_4$ integrin, we examined if treatment with EGF could induce tyrosine phosphorylation of $\alpha_6\beta_4$ in cultured epithelial cells. The human epidermoid carcinoma A431 cells, which express high levels of the EGFR, were serum starved and either left untreated or exposed to EGF. Immunoprecipitation with the anti-β_4 mAb 3E1 followed by immunoblotting with anti-phosphotyrosine (anti-P-Tyr) antibodies indicated that treatment with EGF causes significant tyrosine phosphorylation of the β_4 subunit, suggesting that $\alpha_6\beta_4$ is a direct or indirect target of the EGFR (Fig. 1 *A*). To explore the selectivity of the effect of EGF on β_4 phosphorylation, we asked if exposure to the growth factor also caused tyrosine phosphorylation of β_1 or α_v integrins. We reasoned that this experiment would have provided for a good control, as the cytoplasmic domains of β_1, β_3, β_5, and β_6 contain a conserved sequence motif resembling a major tyrosine autophosphorylation site in the EGFR (Hynes, 1992). As shown in Fig. 1 *A*, immunoprecipitation with the anti-β_1 mAb BV7 or an anti-α_v cytoplasmic domain serum followed by immunoblotting with anti-P-Tyr antibodies showed that exposure to EGF does not induce tyrosine phosphorylation of β_1 or α_v containing integrins (Fig. 1 *A*). This result suggests that the effect of EGF on β_4 phosphorylation is selective. Experiments of [^{32}P]orthophosphate labeling and phosphoamino acid analysis were performed to confirm the ability of EGF to induce tyrosine phosphorylation of β_4. The results indicated that the β_4 subunit is constitutively phosphorylated on serine, and it becomes phosphorylated on tyrosine residues in response to EGF treatment (Fig. 1 *B*). Taken together, these results demonstrate that the β_4 subunit is phosphorylated on tyrosine residues in cells exposed to EGF.

Immunoblotting with anti-phosphotyrosine antibodies indicated that the phosphorylation of β_4 induced by EGF is dose dependent. In A431 cells, we detected a significant level of β_4 phosphorylation in response to as little as 10 ng/ml EGF, and maximal phosphorylation in response to 250 ng/ml EGF (Fig. 2 *A*). The results of time course experiments indicated that the phosphorylation of β_4 induced by EGF in A431 cells follows a biphasic kinetics characterized by a first rapid peak occurring at 2 min and a second one at ~120 min from the initial challenge (Fig. 2 *B*). The decline in β_4 phosphorylation observed at 4 and 8 min after the initial stimulus may be related to the internalization of EGF receptor, a phenomenon that occurs rapidly after ligand binding (Beguinot et al., 1984). This interpretation is supported by the observation that the second peak of β_4 phosphorylation induced by EGF occurs at a time when the downregulation of the EGF receptor has already subsided (Teslenko et al., 1987). The stoichiometry of EGF-induced β_4 phosphorylation was estimated in A431 cells treated for 20 min with 50 ng/ml EGF. After extraction, the tyrosine-phosphorylated integrin was separated from the nonphosphorylated one by affinity chromatography on the anti-P-Tyr mAb 1G2, and both fractions were subjected to immunoblotting with anti-β_4 antibodies (not shown). Densitometric analysis of the results indicated that 83% of the total β_4 subunit had bound to the anti-phosphotyrosine affinity column. From these experiments, we concluded that the tyrosine phosphorylation of β_4 induced by EGF in A431 cells is rapid, dose dependent, and characterized by a high stoichiometry.

We next wondered if treatment with EGF caused tyrosine phosphorylation of β_4 also in normal epithelial cells, which express lower levels of the EGFR than A431 cells. As shown in Fig. 2 *C* (*left*), treatment of primary human keratinocytes with 10 ng/ml EGF caused significant tyrosine phosphorylation of the β_4 subunit. A similar result was obtained with rat epithelial 804G cells expressing moderate levels of recombinant human EGFR (35 times lower than the endogenous EGFR in A431 cells) (Fig. 2 *C*,

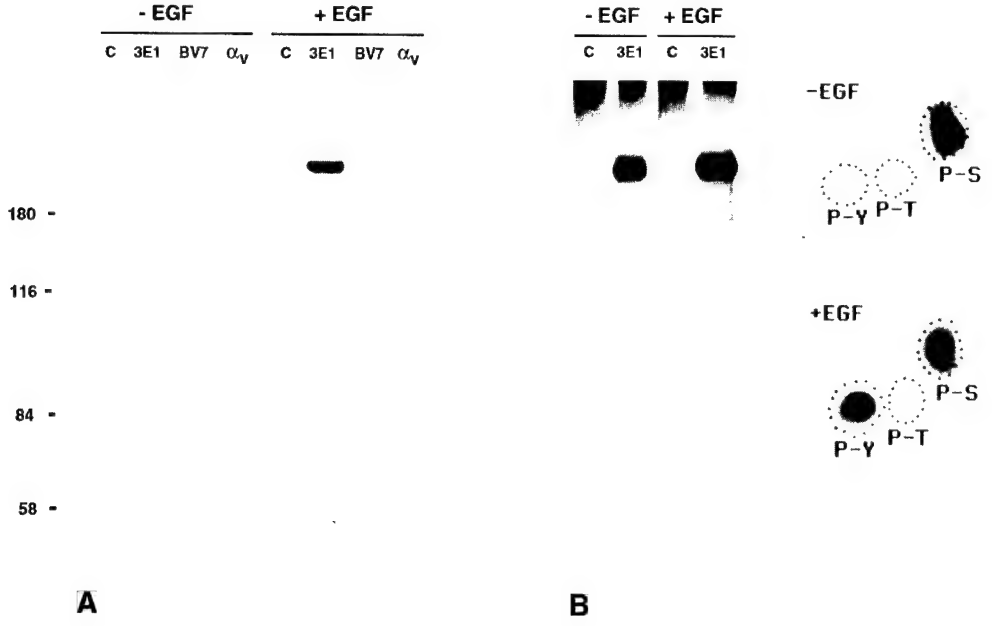


Figure 1. EGF-mediated tyrosine phosphorylation of the β_4 subunit. (*A*) A431 cells were serum starved and either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti–mouse IgGs, anti-human β_4 mAb 3E1, anti-human β_1 mAb BV7, or anti-α_v cytoplasmic domain serum, and then probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (*B*) A431 cells were serum starved, metabolically labeled with [^{32}P]orthophosphate, and then either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti–mouse IgGs or anti-human β_4 mAb 3E1. The radioactive bands corresponding to β_4 were subjected to phosphoamino acid analysis.

right). These results indicate that EGF induces tyrosine phosphorylation of β_4 in cells that express moderate levels of EGFR, including primary epithelial cells.

To explore the mechanism by which the EGFR induces tyrosine phosphorylation of the β_4 subunit, we examined the ability of immunopurified EGFR to phosphorylate in vitro the $\alpha_6\beta_4$ integrin or fusion proteins reproducing the β_4 cytoplasmic domain. Despite undergoing significant autophosphorylation, the EGFR only weakly phosphorylated these potential substrates in in vitro assay (unpublished results). We also wondered if the EGFR and $\alpha_6\beta_4$ stably interacted in A431 cells, but coimmunoprecipitation experiments performed under mild detergent conditions failed to demonstrate a specific association of the two molecules (unpublished results). Although not conclusive, the results of these experiments are consistent with the hypothesis that in vivo the EGFR does not directly phosphorylate β_4, but rather activates a signaling pathway that causes its phosphorylation.

EGF-mediated Tyrosine Phosphorylation of β_4 Requires Ligation of the Integrin by Extracellular Matrix Ligands or Antibodies

To examine if the ability of EGF to induce tyrosine phosphorylation of β_4 is influenced by cell adhesion, A431 cells were detached from the culture substratum, either kept in suspension or plated onto uncoated culture dishes for various times, and then treated with EGF. As shown in Fig. 3 *A*, in the absence of EGF treatment, no tyrosine phosphorylation of β_4 was detected in both suspended and stably adherent cells. Treatment with EGF did not result in tyrosine phosphorylation of β_4 in suspended cells. In contrast, the growth factor induced significant phosphorylation of β_4 in cells that had been plated on the culture dish for $\geq$4 h and were stably adherent. The ability of EGF to induce tyrosine phosphorylation of β_4 did not depend on cell-to-cell contact, as sparse and confluent cells were equally susceptible to the effect of the growth factor. Immunofluorescence experiments indicated that by 4 h of plating the $\alpha_6\beta_4$ integrin had already redistributed to the basal cell surface, presumably in response to extracellular matrix ligands deposited onto the culture substratum during adhesion (data not shown). These observations suggest a correlation between the recruitment of $\alpha_6\beta_4$ to the basal cell surface during adhesion and its susceptibility to EGF-mediated tyrosine phosphorylation.

To examine the hypothesis that the tyrosine phosphorylation of β_4 induced by EGF requires ligation of the integrin by extracellular matrix ligand, A431 cells were plated on dishes coated with the $\alpha_6\beta_4$ ligands laminin 5 and laminin 4 or the control ligand fibronectin, and then treated with EGF. Coating concentrations were adjusted so as to obtain the same extent of cell adhesion and spreading at 30 min. As shown in Fig. 3 *B*, plating of the cells on laminin 5 and 4 rendered the β_4 subunit fully susceptible to EGF-mediated phosphorylation. In contrast, plating on fibronectin had a more modest effect. The effect of EGF on β_4 phosphorylation was maximal during initial adhesion to laminins and then declined. In accordance with the observation that A431 cells adhere with a faster kinetics to laminin 5 than to laminin 4, the peak of β_4 phosphorylation occurred earlier on laminin 5 than on laminin 4. Plating A431 cells onto laminin 5 in the absence of EGF also induced tyrosine phosphorylation of β_4, but this phosphorylation was of lower level and occurred with a slower kinet-

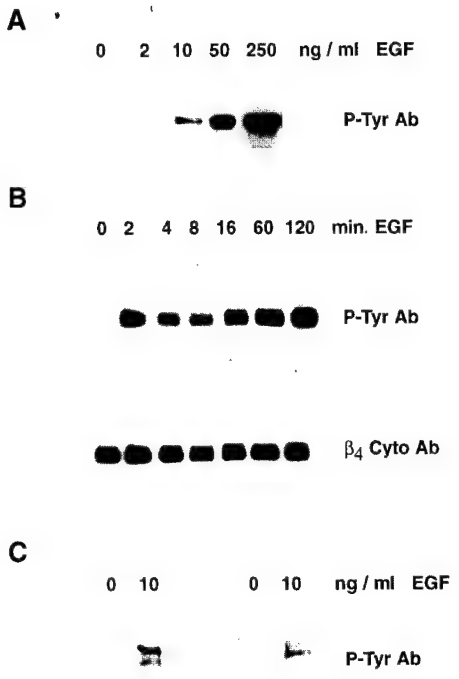


Figure 2. Dose dependence and kinetics of EGF-mediated tyrosine phosphorylation of β_4. Serum-starved A431 cells were treated for 20 min with the indicated concentrations of EGF (*A*), or treated with 50 ng/ml EGF for the indicated times (*B*). After immunoprecipitation with the 3E1 mAb, the samples were probed by immunoblotting with polyclonal anti-P-Tyr or anti-β_4 cytoplasmic domain antibodies. Growth factor–starved primary human keratinocytes (*C*, *left*) and 804G cells expressing a recombinant EGFR (*C*, *right*) were treated with the indicated concentrations of EGF for 15 min. Immunoprecipitation was with anti-β_4 cytoplasmic domain antibodies and immunoblotting with polyclonal anti-P-Tyr antibodies.

ics than in the presence of EGF (Mainiero et al., 1995). Control experiments revealed that the ability of EGFR to undergo autophosphorylation, as well as to induce tyrosine phosphorylation of several cellular substrates, was similar in cells freshly plated on each one of the extracellular matrix proteins tested, including fibronectin (Fig. 3 *C*). These results are consistent with the notion that ligand binding to $\alpha_6\beta_4$ is required for optimal tyrosine phosphorylation of β_4 in response to EGF stimulation.

Since the A431 cells express at least another integrin, $\alpha_3\beta_1$, capable of binding to laminin 5 and possibly to laminin 4, we wished to obtain direct evidence that ligation of $\alpha_6\beta_4$ at the cell surface is required for optimal phosphorylation of β_4. A431 cells were plated for 60 min on the control ligand fibronectin, and then incubated for different times with polystyrene beads coated with the anti-β_4 mAb 3E1, the anti-β_1 mAb TS2/16, or the control anti-MHC mAb W6.32. As shown in Fig. 4, treatment with EGF caused significant tyrosine phosphorylation of β_4 in cells exposed for 10 min to the anti-β_4 beads, but not in cells treated with anti-β_1 or anti-MHC beads. Incubation with soluble 3E1 mAb produced a very modest effect. Control experiments indicated that treatment of A431 cells with anti-β_1 beads in the absence of EGF induces, as expected, a significant tyrosine phosphorylation of $p125^{FAK}$ (data not shown). These results suggest that $\alpha_6\beta_4$ must be oligomerized at the cell surface to be susceptible to EGF-mediated phosphorylation.

Interestingly, while EGF could consistently induce significant tyrosine phosphorylation of β_4 in cells that had been plated onto a plastic culture substratum for 4 h or more (Fig. 3 *A*), maximal phosphorylation of β_4 occurred only transiently in cells plated on laminin 5 and 4 (Fig. 3 *B*) or incubated with anti-β_4 beads (Fig. 4). The transient nature of the effect induced by initial ligation of $\alpha_6\beta_4$ on tyrosine phosphorylation of β_4 may be explained by the pre-

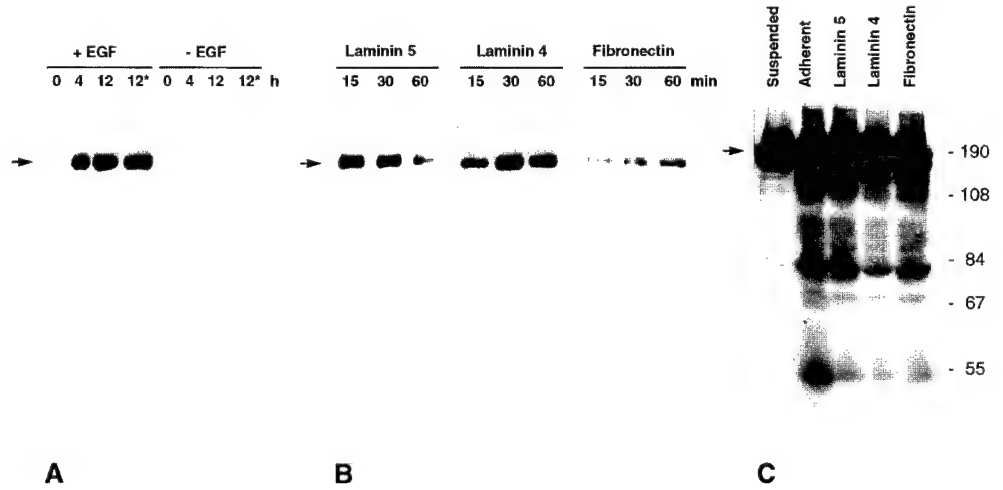


Figure 3. EGF-mediated tyrosine phosphorylation of β_4 requires extracellular matrix ligand binding. (*A*) A431 cells were detached, resuspended in complete medium, and either kept in suspension or replated at low density onto uncoated dishes for 4 or 12 h. Where indicated by the asterisk, the cells were plated at high density and reached confluence by 12 h. The cells were either left untreated or stimulated with 250 ng/ml EGF for 20 min, and then immunoprecipitated with the 3E1 mAb. Samples were probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (*B*) A431 cells were serum starved, detached, and then replated in serum-free medium on dishes coated with laminin 5 matrix, 10 μg/ml laminin 4, or 10 μg/ml fibronectin for the indicated times. In all cases, the cells were treated with 50 ng/ml EGF for 5 min, and then immunoprecipitated with the 3E1 mAb. Samples were probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (*C*) A431 cells were serum starved, detached, and then either kept in suspension or replated in serum-free medium on dishes coated with laminin 5 matrix, 10 μg/ml laminin 4, or 10 μg/ml fibronectin for 30 min. In all cases, the cells were treated with 50 ng/ml EGF for 5 min. Total proteins were probed by immunoblotting with polyclonal anti-P-Tyr antibodies.

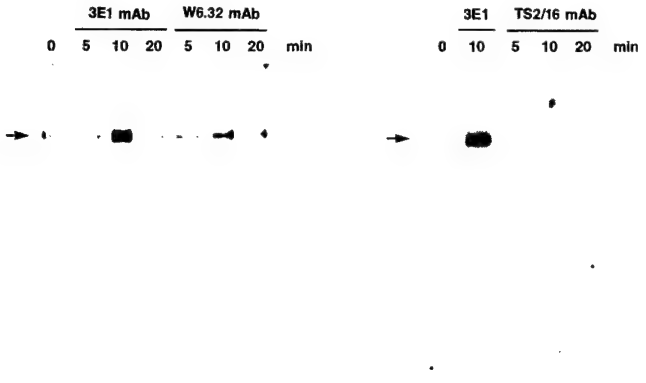


Figure 4. EGF-mediated tyrosine phosphorylation of β_4 requires ligation of the integrin. A431 cells were serum starved, detached, replated in serum-free medium on dishes coated with 10 μg/ml fibronectin for 60 min, and then incubated for the indicated times with beads coated with the anti-β_4 mAb 3E1, the anti-β_1 mAb TS2/16, or the control anti-MHC mAb W6.32. In all cases, the cells were treated with 50 ng/ml EGF for 5 min, and then immunoprecipitated with the 3E1 mAb. The samples were probed by immunoblotting with polyclonal anti-P-Tyr antibodies.

vious observation that tyrosine phosphorylation of β_4 is negatively regulated by tyrosine phosphatases (Mainiero et al., 1995). Thus, although ligation of $\alpha_6\beta_4$ can activate a tyrosine kinase responsible for β_4 phosphorylation and synergize with the effect of EGF, the subsequent activation of tyrosine phosphatases able to reverse the phosphorylation of β_4 is likely to antagonize the effect of EGF. Taken together, these results suggest that clustering of $\alpha_6\beta_4$ induced by extracellular matrix ligands is required for optimal phosphorylation of the β_4 subunit in response to EGF stimulation. They further suggest that, depending on the timing, ligand binding to $\alpha_6\beta_4$ can either synergize or antagonize with a signal from the EGFR to induce tyrosine phosphorylation of β_4.

EGF Induces Phosphorylation of Multiple β_4 Tyrosine Residues

Phosphopeptide mapping experiments were performed to analyze the β_4 sites phosphorylated in response to EGF treatment. Since cross-linking of $\alpha_6\beta_4$ by antibodies or plating on laminin 5 did not induce a level of tyrosine phosphorylation of β_4 sufficient for high resolution mapping, the sites phosphorylated in response to EGF were compared to those phosphorylated in response to pervanadate. Previous results have shown that treatment with pervanadate results in phosphorylation of multiple β_4 residues, including the β_4 TAM and presumably also the Shc binding sites, since pervanadate can induce association of $\alpha_6\beta_4$ with Shc (Mainiero et al., 1995). A431 cells were metabolically labeled with [^{32}P]orthophosphate, and then either left untreated or stimulated with EGF or pervanadate. After immunoprecipitation, the β_4 subunit was digested with Staphylococcus V8 protease, and the resulting peptides were separated by bidimensional TLC. As shown in Fig. 5 *A*, the β_4 subunit from unstimulated cells was resolved in a number of phosphopeptides (S1–S10). In accordance with the observation that β_4 is phosphorylated constitutively on serine residues (Fig. 5 *B*), phosphoamino acid analysis indicated that these peptides contained only phosphoserine. Treatment with EGF resulted in the appearance of a number of additional phosphopeptides (Y1–Y8) (Fig. 5 *B*), and phosphoamino acid analysis of several of them (Y1–Y6) confirmed that they contained exclusively phosphotyrosine. These results indicate that exposure to EGF results in phosphorylation of multiple tyrosine residues in the β_4 cytoplasmic domain. The phosphopeptide map of β_4 from pervanadate-treated cells was similar, but not identical, to that of β_4 from EGF-stimulated cells. It contained the peptides S1–S10 and Y1–Y8, but also an additional phosphotyrosine-containing peptide, Y9. Furthermore, the intensity of the spot corresponding to phosphopeptide Y1 was much larger in pervanadate than in EGF-treated cells, and conversely, phosphopeptide Y6 was more intensely labeled in EGF than in pervanadate-treated cells (Fig. 5 *C*). Previous experiments of site-directed mutagenesis and phosphopeptide mapping of β_4 from pervanadate-treated cells have indicated that peptide Y5 contains tyrosine 1440, the COOH-terminal element of the TAM, and have provided circumstantial evidence that peptide Y2 contains tyrosine 1422, the NH_2-terminal element of the TAM (Mainiero et al., 1995). Since exposure to EGF resulted in the appearance of phosphopeptides Y5 and Y2, we concluded that EGF induces the phosphorylation of multiple tyrosine residues in β_4 and that these include the COOH-terminal, and possibly the NH_2-terminal, element of the TAM.

EGF-mediated Tyrosine Phosphorylation of β_4 Does Not Result in Recruitment of the Adaptor Proteins Shc and Grb2

To examine if EGF-mediated tyrosine phosphorylation of β_4 results in association of the adaptor protein Shc to $\alpha_6\beta_4$, A431 cells were either incubated with anti-β_4 beads in suspension or treated with EGF while adherent. The resulting extracts were immunoprecipitated with anti-β_4 antibodies and probed by immunoblotting with anti-β_4 and anti-Shc antibodies. As shown in Fig. 6 *A*, ligation of $\alpha_6\beta_4$ led to recruitment of Shc. In contrast, treatment with EGF did not result in association of this adaptor molecule to $\alpha_6\beta_4$. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-β_4 antibodies confirmed that EGF stimulation does not result in recruitment of Shc to $\alpha_6\beta_4$ (Fig. 6 *B*). Control experiments indicated that a certain amount of the adaptor molecule remained available in the cytoplasm of EGF-treated cells (not shown; see also Fig. 6 *C*). The results of these experiments suggest that EGF does not induce phosphorylation of the Shc binding sites in β_4.

The inability of EGF to induce association of Shc with the $\alpha_6\beta_4$ integrin raises the possibility that the EGFR and $\alpha_6\beta_4$ may, when simultaneously ligated, compete for this adaptor molecule in vivo. To explore this possibility, we examined the effect of EGF on the recruitment of Shc to activated $\alpha_6\beta_4$. As shown in Fig. 6 *C*, the amount of Shc coimmunoprecipitated with $\alpha_6\beta_4$ was lower in cells stimulated with anti-β_4 beads and EGF than in cells treated only with anti-β_4 beads. The inhibitory effect of EGF was especially evident in cells that had been incubated with anti-β_4 beads for 5 or 10 min, irrespective of whether the EGF was

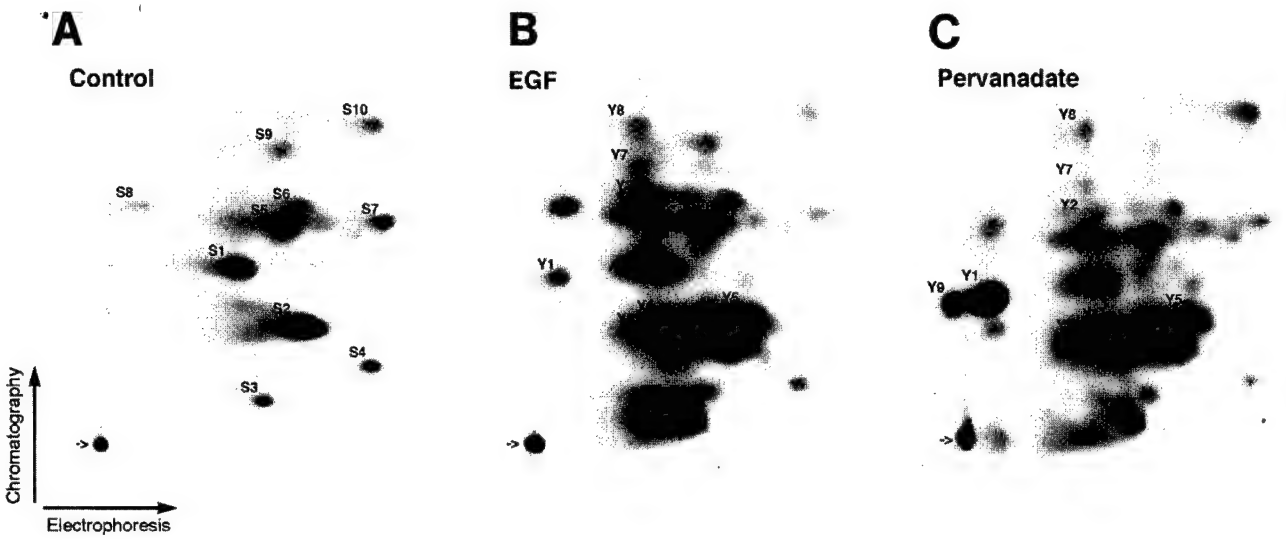


Figure 5. Phosphorylation of multiple β_4 tyrosine residues in response to EGF. A431 cells were metabolically labeled with [^{32}P]orthophosphate and left untreated (*A*), stimulated with 250 ng/ml EGF for 5 min (*B*), or with 500 μM pervanadate for 10 min (*C*). After immunoprecipitation with the 3E1 mAb and separation by SDS-PAGE, the radioactive bands corresponding to β_4 were subjected to V8 protease digestion, and the resulting phosphopeptides were separated by bidimensional TLC.

applied together with the beads or before the beads. In addition to indicating that a certain amount of Shc remains available for binding to $\alpha_6\beta_4$ in EGF-treated cells, these results indicate that the EGFR and $\alpha_6\beta_4$ integrin compete

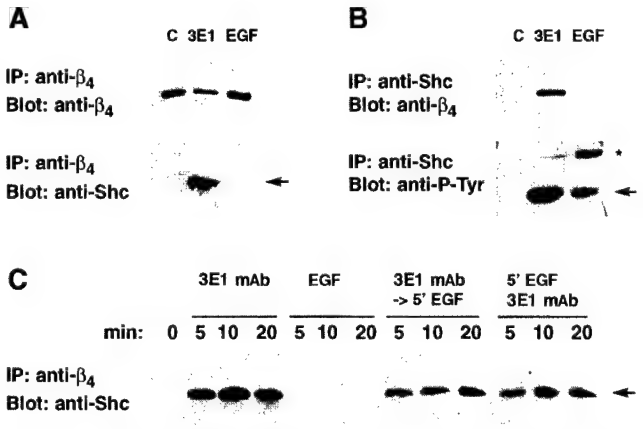


Figure 6. EGF interferes with the recruitment of Shc to $\alpha_6\beta_4$. (*A*) A431 cells were serum starved and either incubated for 10 min in suspension with polystyrene beads coated with the anti-β_4 mAb 3E1 or treated for 5 min while adherent with 200 ng/ml EGF. Control cells consisted of suspended cells left untreated. Equal amounts of total proteins were immunoprecipitated with anti-β_4 cytoplasmic peptide serum and probed by immunoblotting with the same antiserum (*top*) or anti-Shc mAb (*bottom*). (*B*) A431 cells were treated as above, but immunoprecipitated with anti-Shc polyclonal antibodies and probed with either anti-β_4 cytoplasmic peptide serum (*top*) or the anti-P-Tyr mAb PY20 (*bottom*). (*C*) A431 cells were serum starved, detached and replated in serum-free medium on dishes coated with 10 μg/ml fibronectin for 60 min. They were then incubated for the indicated times with 3E1 mAb-coated beads, 50 ng/ml EGF, or 3E1 mAb-coated beads followed or preceded by a 5-min exposure to 50 ng/ml EGF. The extracts were immunoprecipitated with anti-β_4 cytoplasmic peptide antibody and probed by immunoblotting with anti-Shc mAb.

for this adaptor molecule in cultured cells, raising the possibility that the ability of $\alpha_6\beta_4$ to link to the *ras* pathway may be suppressed by EGF-dependent signals in vivo.

Disruption of Hemidesmosomes by EGF

To examine the effect of EGF on the ability of $\alpha_6\beta_4$ to associate with the hemidesmosomal cytoskeleton, we elected to use the rat 804G bladder epithelial cells, which form hemidesmosomes in vitro. Since these cells express very low levels of the EGFR, we used cell lines expressing moderate levels of human EGFR from cDNA. Immunoblotting analysis of total proteins with anti-P-Tyr antibodies indicated that exposure of the EGFR-transfected cells to EGF resulted in tyrosine phosphorylation of the recombinant EGFR and of several of its cellular substrates, including the β_4 subunit (data not shown and Fig. 2 *C*, *right*).

The EGFR-transfected 804G cells were starved, and then either left untreated or treated for various times with 100 ng/ml EGF. Immunofluorescent analysis revealed that while in control cells, $\alpha_6\beta_4$ and the BPAG 2 were concentrated at the basal cell surface within Triton X-100–resistant, "Swiss cheese"-like structures corresponding to hemidesmosomes (Fig. 7, *a* and *b*); in cells treated with EGF, these molecules had undergone a profound redistribution and were no longer detected in association with these structures (Fig. 7, *d* and *e*). To confirm the physiological significance of these observations, we examined the effect of EGF on the hemidesmosome-like structures formed by normal human primary keratinocytes in culture. As shown in Fig. 7 (*c* and *f*), treatment with EGF resulted in loss of hemidesmosomal staining also in these cells, suggesting that disassembly of hemidesmosomes may be one of the physiological consequences of activation of the EGFR in primary epithelial cells. Immunofluorescent analysis of EGFR transfected 804G cells treated for various times with EGF indicated that the effect of the growth factor on hemidesmosomes was already significant after 1 h and

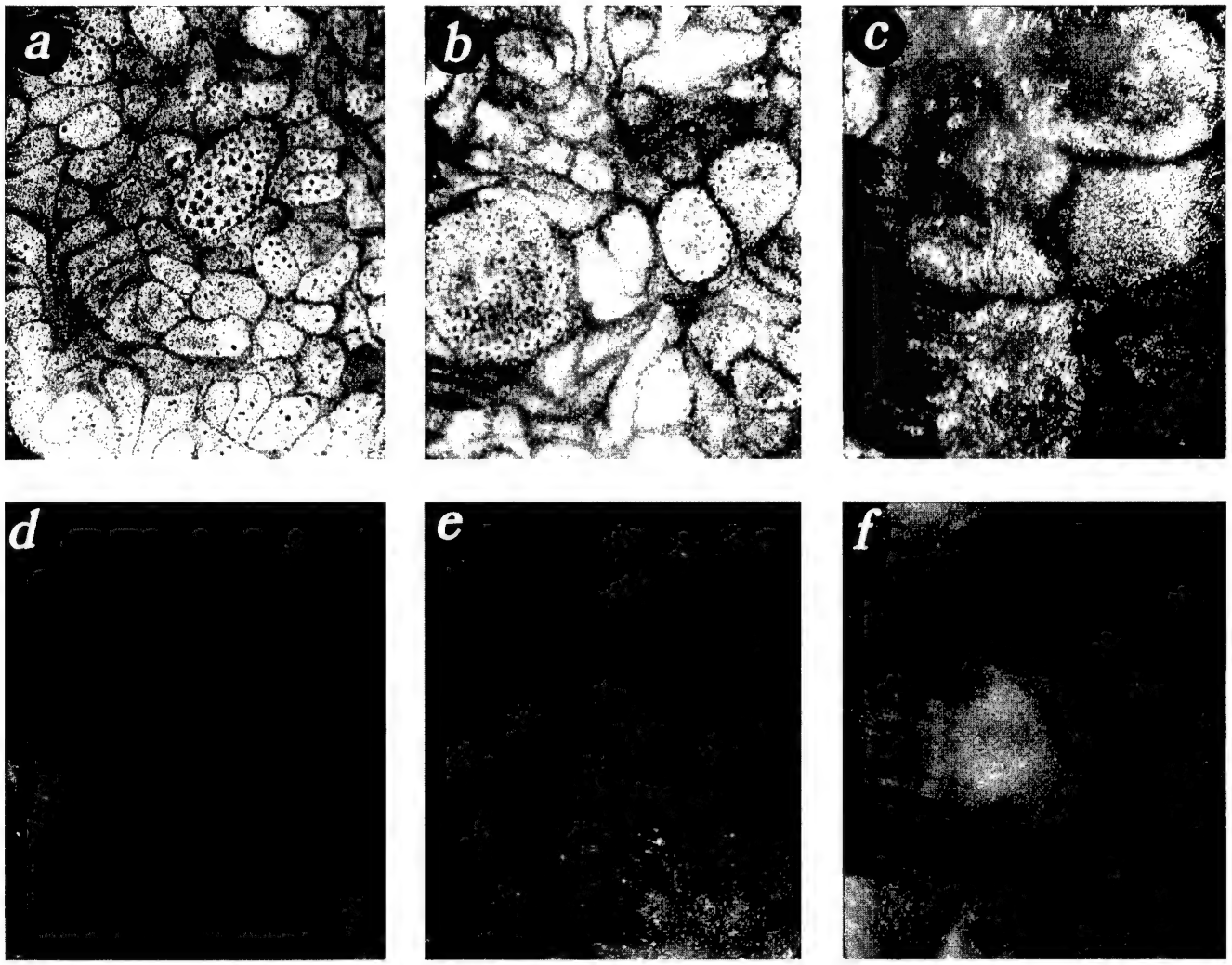


Figure 7. Disruption of hemidesmosomes by EGF. EGFR-transfected 804G cells (*a*, *b*, *d*, and *e*) and primary human keratinocytes (*c* and *f*) were cultured on glass coverslips for 48 h, serum starved, and either left untreated (*a–c*) or treated with 100 ng/ml EGF for 12 h (*d–f*). After extraction with 0.2% Triton X-100, the cells were fixed and stained with anti-BPAG 2 antibodies (*a* and *d*), anti-β_4 cytoplasmic peptide serum (*b* and *e*), or the anti-β_4 mAb 3E1 (*c* and *f*) followed by FITC-labeled affinity-purified secondary antibodies.

complete by 12 h of treatment (Fig. 8, *a–e*). Dose-dependency experiments indicated that 25 ng/ml of EGF were sufficient to induce a significant effect on hemidesmosomes (data not shown). The effect of EGF was specific as PDGF did not cause any change in hemidesmosome staining (Fig. 8 *f*). Similar results were obtained with three independent clones of EGFR-transfected 804G cells. These observations indicate that EGF treatment causes disruption of hemidesmosomes.

The ability of EGF to induce hemidesmosome disassembly was unexpected because phosphopeptide mapping had indicated that the β_4 TAM, which mediates a signaling event required for the association of $\alpha_6\beta_4$ with hemidesmosomes (Mainiero et al., 1995), is phosphorylated in response to EGF. We wondered if the effect of EGF on hemidesmosomes was caused by its ability to downregulate ligand binding to $\alpha_6\beta_4$ by a mechanism of inside-to-outside signaling. The effect of EGF treatment on the adhesion of EGFR-transfected 804G cells to laminin 4 and 5 was therefore examined. To block β_1-dependent adhesion, the cells were plated on the two extracellular matrix proteins in the presence of inhibitory anti-β_1 antibodies. As shown in Fig. 9 , the extent to which the EGFR-transfected 804G cells adhered to laminin 4 and 5 was not significantly changed after treatment with EGF. A similar result was obtained with A431 cells (not shown). These results indicate that exposure to EGF does not cause a significant change in ligand binding to $\alpha_6\beta_4$, thus suggesting that the deterioration of hemidesmosomes observed in EGF-treated cells is not caused by a downregulation of ligand binding. Together with the observation that EGF induces phosphorylation of the β_4 TAM, these data suggest the hypothesis that EGF-dependent signals suppress the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton by interfering with the functioning of signaling and cytoskeletal molecules downstream of the β_4 TAM.

Increased $\alpha_6\beta_4$-dependent Cell Migration in Response to EGF

To determine if the apparent disruption of hemidesmosomes caused by EGF correlates with a change in $\alpha_6\beta_4$-

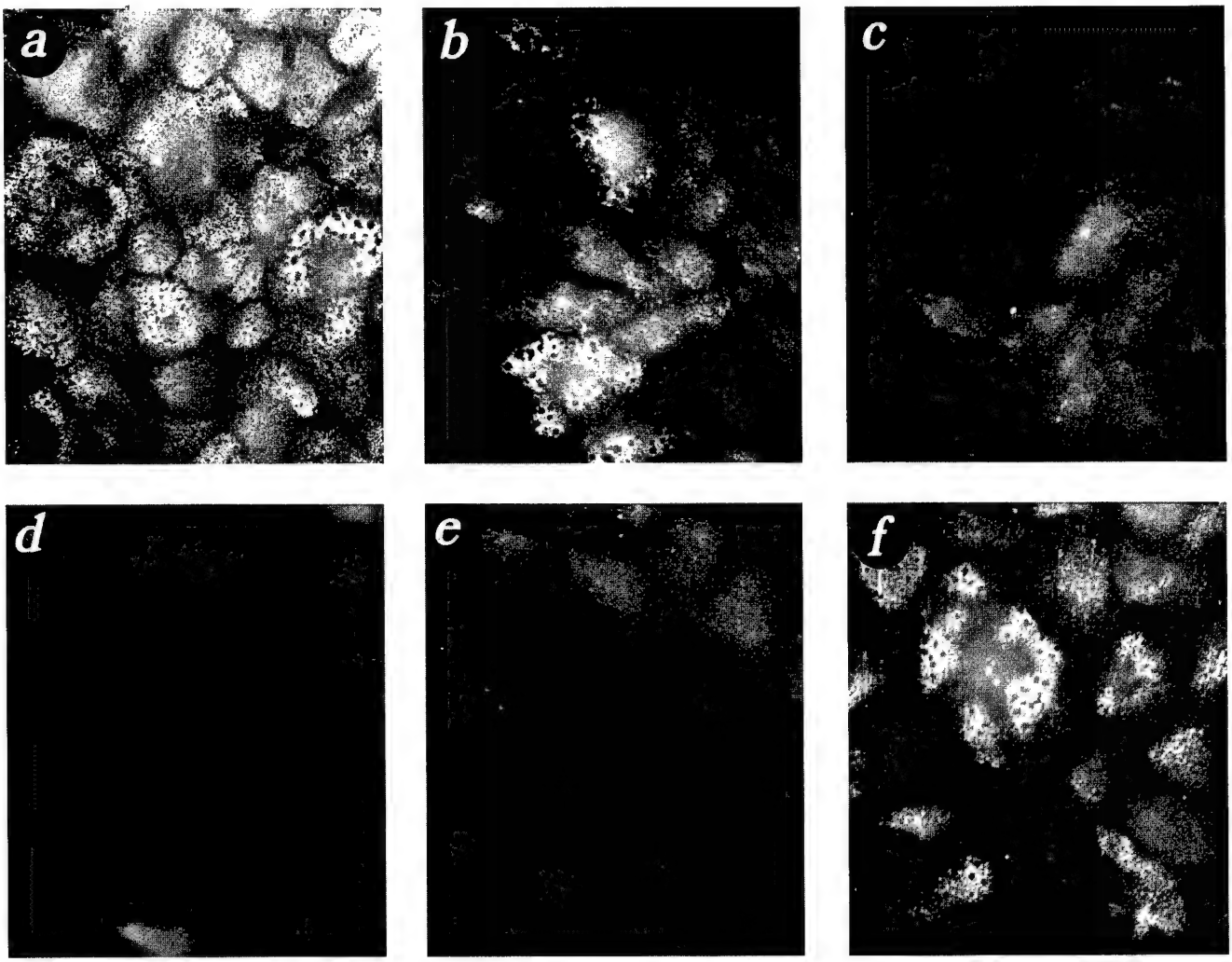


Figure 8. Kinetics and specificity of EGF-induced hemidesmosome disruption. EGFR-transfected 804G cells were cultured on glass coverslips for 48 h, serum starved, and either left untreated (*a*) or treated with 100 ng/ml EGF for 30 min (*b*), 1 h (*c*), 3 h (*d*), or 12 h (*e*). As a control, cells were exposed to 5 ng/ml PDGF for 12 h (*f*). After extraction with 0.2% Triton X-100, the cells were fixed and stained with anti-BPAG 2 antibodies followed by FITC-labeled affinity-purified secondary antibodies.

dependent cell migration, we measured the ability of control and EGFR-transfected 804G cells to migrate toward various extracellular matrix components by using a Boyden chamber system. As shown in Fig. 10, treatment with EGF resulted in increased migration of the EGFR-transfected 804G cells toward the two $\alpha_6\beta_4$ ligands laminin 4 and 5, but not the control ligand fibronectin, suggesting that EGF-dependent signals can increase cell migration toward $\alpha_6\beta_4$ ligands. In addition, the basal migration of EGFR-transfected 804G cells toward laminin 4 and 5 was greater than that of control 804G cells. This result suggests that the recombinant EGFR may be partially active in the absence of exogenous ligand in 804G cells, perhaps because these cells secrete EGF or TGF-α. In accordance with this hypothesis, we found that the medium conditioned by 804G cells is capable of stimulating the autophosphorylation of recombinant EGFR expressed in transfected 804G cells (data not shown). Inhibitory anti-β_1 antibodies were able to suppress the migration of unstimulated cells toward laminin 5 by 91 ± 6%, but only inhibited the migration of EGF-treated cells by 11 ± 3%, indicating that the EGF-stimulated migration toward laminin 5 was largely dependent on $\alpha_6\beta_4$ function. This conclusion was also supported by the observation that EGFR-transfected NIH-3T3 fibroblasts, which do not express $\alpha_6\beta_4$, did not respond to EGF with increased migration toward laminin 4 (Fig. 10, *top*). Finally, the effect of EGF was specific, since it was not observed in response to PDGF or with the control 804G cells in response to EGF. Taken together, these results indicate that EGF specifically upregulates $\alpha_6\beta_4$-dependent migration toward laminins.

Discussion

Several observations suggest that $\alpha_6\beta_4$- and growth factor–dependent signals may cooperate to control epidermal cell proliferation and migration. In stratified epithelia, such as the epidermis, $\alpha_6\beta_4$ mediates the interaction of basal keratinocytes with the basement membrane (Kajiji et al., 1989), and there is evidence indicating that these cells have to remain in contact with this extracellular matrix to maintain their proliferative potential (Green, 1977; Hall and

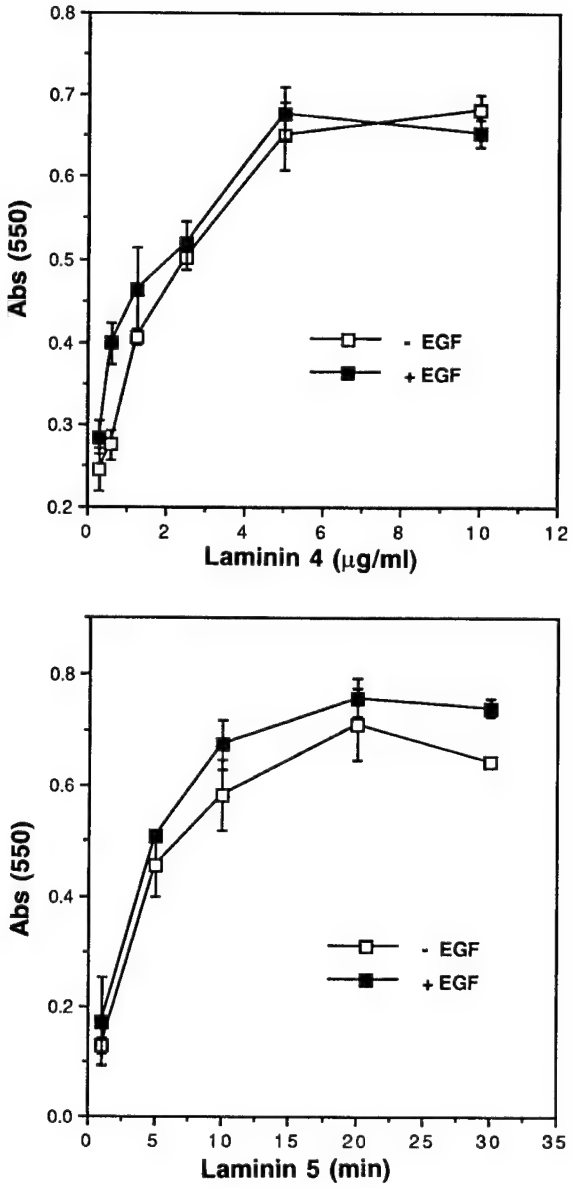


Figure 9. EGF does not affect $\alpha_6\beta_4$-dependent cell adhesion. EGFR-transfected 804G cells were starved and either left untreated or stimulated with 100 ng/ml EGF. The cells were plated in the presence of inhibitory anti-β_1 antibodies on dishes coated with the indicated amounts of laminin 4 for 60 min (*top*) or on laminin 5 matrix-coated dishes for the indicated times (*bottom*).

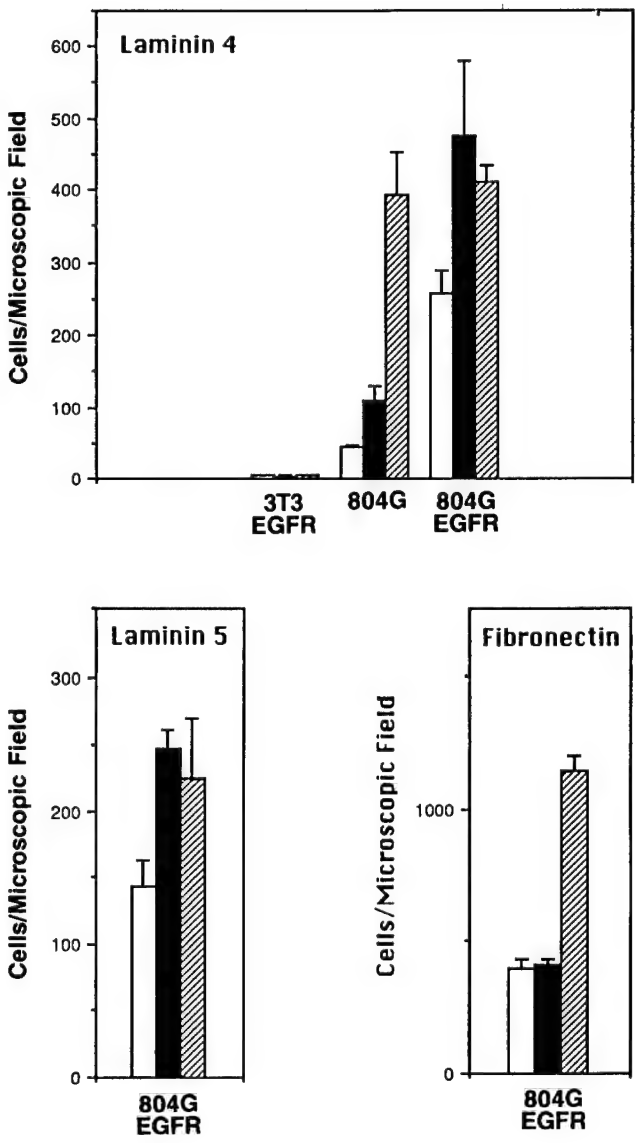


Figure 10. EGF stimulates $\alpha_6\beta_4$-dependent cell migration. The indicated cell lines were allowed to migrate toward laminin 4 for 48 h, laminin 5 for 12 h, and fibronectin for 48 h in the presence of control medium (*open bars*), 50 ng/ml EGF (*closed bars*), or 5 ng/ml PDGF (*hatched bars*).

Watt, 1989). Furthermore, the coincident expression of $\alpha_6\beta_4$ and laminins by keratinocytes migrating into corneal wounds suggests a role for $\alpha_6\beta_4$-mediated migration during the reepithelialion of wounds (Kurpakus et al., 1991). Prompted by the prominent role of EGF and transforming growth factor α in controlling keratinocyte growth and migration (Rheinwald and Green, 1977; Barrandon and Green, 1987), and by the coincident expression of $\alpha_6\beta_4$ and EGFR in basal keratinocytes in vivo (Green et al., 1987; Kajiji et al., 1989), we have examined the effect of EGFR activation on the intracellular functions of $\alpha_6\beta_4$. Our results indicate that EGF-dependent signals have a complex effect on $\alpha_6\beta_4$ function: they cause tyrosine phosphorylation of β_4 without promoting the association of Shc, induce disassembly of hemidesmosomes, and upregulate cell migration on laminins.

In this study, we provide direct evidence that activation of the EGFR causes tyrosine phosphorylation of the β_4 subunit. This phosphorylation is characterized by a rapid kinetics and, at least in A431 cells, by a high stoichiometry. Since we have been unable to obtain evidence that the EGFR efficiently phosphorylate β_4 in vitro, it is our hypothesis that the EGFR does not directly phosphorylate β_4 in vivo, but rather it activates a signaling pathway that results in its phosphorylation. The observation that EGF-mediated phosphorylation of β_4 requires ligation of the integrin by extracellular ligand or antibodies suggests that this phosphorylation event is mediated by an integrin-associated kinase acting in trans. Future studies will be required to determine if $\alpha_6\beta_4$ is indeed an indirect target of

the EGFR and if it is associated with two distinct tyrosine kinases, one activated by EGF and the other by extracellular matrix binding, or with a single tyrosine kinase activated by both stimuli.

The results of phosphopeptide mapping indicate that EGF causes phosphorylation of several distinct β_4 tyrosine residues. Although the majority of the tyrosine phosphorylation sites in β_4 remain to be identified and their function assessed, the complexity of the tyrosine phosphorylation pattern induced by EGF suggests that many $\alpha_6\beta_4$ functions may be regulated by the growth factor. One major intracellular function of $\alpha_6\beta_4$ that is negatively regulated by EGF is the recruitment of the adaptor molecule Shc. Treatment with EGF does not result in the association of $\alpha_6\beta_4$ with Shc and presumably Grb2. In fact, exposure to EGF partially suppresses the recruitment of Shc to the ligated integrin. Although it is possible that EGF causes a conformational change or another posttranslational modification of $\alpha_6\beta_4$ that prevents it from binding to Shc, the most likely explanation of these results is that the growth factor does not induce phosphorylation of the Shc binding motifs in β_4. The observation that the EGFR can compete with $\alpha_6\beta_4$ for the recruitment of Shc is in accordance with the recognized ability of activated EGFR to associate with this adaptor molecule (Pellicci et al., 1992) and suggests that a significant activation of the EGFR may interfere with the ability of ligand-occupied $\alpha_6\beta_4$ to activate signaling in vivo. In contrast, when suboptimally ligated, the EGFR and $\alpha_6\beta_4$ are likely to cooperate with each other to activate the *ras* pathway. This latter prediction may be relevant to understanding anchorage-dependent cell growth in epithelial cells.

The results of our immunofluorescent analysis indicate that treatment with EGF causes disruption of hemidesmosomes in both EGFR-transfected 804G cells and primary human keratinocytes. What is the mechanism by which EGF interferes with the assembly of hemidesmosomes? Our previous studies suggest that the nucleation of hemidesmosomes requires a signal mediated by the β_4 TAM (Mainiero et al., 1995). It is, however, unlikely that the phosphorylation of the TAM is the only $\alpha_6\beta_4$ function necessary for the assembly of hemidesmosomes. Deletion mutagenesis experiments have indicated that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton not only requires the connecting segment, which includes the TAM, but also sequences within the two type III fibronectin-like modules upstream of the connecting segment (Spinardi, L., and F.G. Giancotti, unpublished results). This observation is consistent with the hypothesis that a TAM-dependent signal renders one or more cytoskeletal elements of hemidesmosomes competent for binding to sequences within the first two type III fibronectin-like modules of β_4. Further assembly of hemidesmosomes may then be driven by the cooperative binding of additional cytoskeletal elements. Based on this model, EGF-dependent signals may interfere with the assembly of hemidesmosomes at one or more of several steps. Since EGF does not affect $\alpha_6\beta_4$-mediated adhesion to laminins and does not suppress phosphorylation of the β_4 TAM, the growth factor may interfere with the functioning of one or more signaling or cytoskeletal molecules located downstream of the TAM in the pathway that controls the association of $\alpha_6\beta_4$ with the cytoskeleton. Furthermore, it is possible that EGF induces the phosphorylation of tyrosine residues located within the first two type III fibronectin-like modules of the β_4 tail, thus directly interfering with the association of cytoskeletal molecules. Finally, as the process of hemidesmosome formation is likely to be complex and to require the function of many components in addition to $\alpha_6\beta_4$ and the molecules to which it binds, EGF may disrupt hemidesmosomes by acting on one or more of these additional components.

Most of the previous studies on the regulation of the cytoskeleton by growth factors have focused on the effects of EGF and PDGF on the actin filament system. It has been known for long that these growth factors can induce profound changes in the architecture of the actin cytoskeleton (Bockus and Stiles, 1984; Herman and Pledger, 1985). Recent studies have indicated that they can induce the sequential formation of filopodia, lamellipodia, and focal adhesions, and that these cytoskeletal changes are mediated by a GTPase cascade involving Cdc 42, Rac, and Rho (Nobes and Hall, 1995). Our current observations clearly indicate that EGF can also profoundly affect the keratin filament system, thereby providing evidence for a novel mechanism of cytoskeletal regulation by EGF.

The changes in the association of $\alpha_6\beta_4$ with the cytoskeleton induced by activated EGFR are likely to be significant in both physiological and pathological situations. Several lines of evidence support the notion that hemidesmosomes mediate stable adhesion to the basement membrane (Uitto and Christiano, 1992; Guo et al., 1995; Spinardi et al., 1995). Their disruption may therefore result in a more dynamic interaction with the extracellular matrix. In accordance with this hypothesis, we have observed that the disassembly of hemidesmosomes caused by EGF correlates with an increase in $\alpha_6\beta_4$-dependent cell migration. This observation suggests that the ability of $\alpha_6\beta_4$ to mediate cell migration on laminins can be upregulated by factors that interfere with its association with the hemidesmosomal cytoskeleton. It is well known that EGF and TGF-α can promote the reepithelialization of wounds (Schultz et al., 1991), and it has recently been observed that keratinocytes lose their hemidesmosomes as they migrate into corneal wounds (Gipson et al., 1993). Thus, the ability of activated EGFR to coordinately disassemble hemidesmosomes and increase cell migration on laminins is likely to be important during wound healing. In addition, there is evidence indicating that keratinocytes of patients affected by the skin disease psoriasis overproduce TGF-α (Elder et al., 1989) and that squamous carcinoma cells overexpress the EGFR (Yamamoto et al., 1986; Ozanne et al., 1986). In both pathological situations, the expression of $\alpha_6\beta_4$ is no longer restricted to the basal surface of those cells that abut the basement membrane, but extends suprabasally (Kimmel and Carey, 1986; Pellegrini et al., 1992). Our current results suggest that the loss of $\alpha_6\beta_4$ polarity observed in these diseases may result from the ability of activated EGFR to disrupt the association of the integrin to the hemidesmosomal cytoskeleton. They further suggest that the ability of EGFR to affect the association of $\alpha_6\beta_4$ with the cytoskeleton may contribute to the invasive ability of squamous carcinoma cells.

F. Mainiero and A. Pepe contributed equally to this work. We thank Elis-

abetta Dejana, Eva Engvall, Erkki Ruoslahti, and Jouni Uitto for antibodies, Jossi Schlessinger for the EGFR expression construct and antibodies, members of our laboratory for helpful suggestions, Miki Blumemberg for human primary keratinocytes, and Jan Sap for critical comments on the manuscript.

This work was supported by Public Health Service (PHS) grant R01-CA58976, grant DAMD 17-94-J4306 from the U.S. Army Medical Research and Material Command, and PHS core support grant P30-CA16087. F. Mainiero is supported by a fellowship from the American Italian Foundation for Cancer Research. F.G. Giancotti is a recipient of awards from the Lucille P. Markey and Irma T. Hirschl Charitable Trusts.

Received for publication 28 November 1995 and in revised form 16 March 1996.

References

Argraves, W.S., R. Pytela, S. Suzuki, J.L. Millan, M.D. Pierschbacher, and E. Ruoslahti. 1986. cDNA sequences from the α subunit of the fibronectin receptor predict a transmembrane domain and a short cytoplasmic peptide. *J. Biol. Chem.* 261:12922–12924.

Arroyo, A.G., P. Sánchez-Mateos, M.R. Campanero, I. Martín-Padura, E. Dejana, and F. Sanchez-Madrid. 1992. Regulation of VLA integrin–ligand interactions through the β_1 subunit. *J. Cell Biol.* 117:659–670.

Barrandon, Y., and H. Green. 1987. Cell migration is essential for substained growth of keratinocyte colonies: the roles of transforming growth factor-α and epidermal growth factor. *Cell* 50:1131–1137.

Beguinot, L., R.M. Lyall, M.C. Willingham, and I. Pastan. 1984. Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. *Proc. Natl. Acad. Sci. USA.* 81:2384–2388.

Burridge, K., C.E. Turner, and L.H. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119:893–903.

Bockholt, S.M., and K. Burridge. 1993. Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. *J. Biol. Chem.* 268: 14565–14567.

Bockus, B.J., and C.D. Stiles. 1984. Regulation of cytoskeletal architecture by platelet-derived factor, insulin and epidermal growth factor. *Exp. Cell Res.* 153:186–197.

Boyle, W.J., P. Van Der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoaminoacid analysis by two-dimensional separation on thin layer cellulose plates. *Methods Enzymol.* 201:110–149.

Carter, W.G., P. Kaur, S.G. Gil, P.J. Gahr, and E.A. Wayner. 1990. Distinct functions for integrins $\alpha_3\beta_1$ in focal adhesions and $\alpha_6\beta_4$/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* 111:3141–3154.

Chen, H.-C., and J.-L. Guan. 1994. Stimulation of phosphatidylinositol 3′-kinase association with focal adhesion kinase by platelet-derived growth factor. *J. Biol. Chem.* 269:31229–31233.

Chen, J., J. Kim, K. Zhang, Y. Sarret, K. Wynn, R. Kramer, and D. Woodley. 1993. Epidermal growth factor (EGF) promotes human keratinocytes locomotion on collagen by increasing the α_2 integrin subunit. *Exp. Cell Res.* 209: 216–223.

Chong, L.D., A. Traynor-Kaplan, G.M. Bokoch, and M.A. Schwartz. 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell.* 79:507–513.

Elder, J.T., G.J. Fisher, P.B. Lindquist, G.L. Bennett, M.R. Pittelkow, R.J. Coffey, L. Ellingsworth, R. Derynck, and J.J. Voorhees. 1989. Overexpression of transforming growth factor alpha in psoriatic epidermis. *Science (Wash. DC).* 243:811–814.

Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Giancotti, F.G., and F. Mainiero. 1994. Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta.* 1198:47–64.

Giancotti, F.G., G. Tarone, K. Knudsen, C. Damsky, and P.M. Comoglio. 1985. Cleavage of a 135 kD cell surface glycoprotein correlates with loss of fibroblast adhesion to fibronectin. *Exp. Cell Res.* 156:182–190.

Giancotti, F.G., P.M. Comoglio, and G. Tarone. 1986. Fibronectin–plasma membrane interaction in the adhesion of hemopoietic cells. *J. Cell Biol.* 103: 429–437.

Giancotti, F.G., M.A. Stepp, S. Suzuki, E. Engvall, and E. Ruoslahti. 1992. Proteolytic processing of endogenous and recombinant β_4 integrin subunit. *J. Cell Biol.* 118:951–959.

Giancotti, F.G., L. Spinardi, F. Mainiero, and R. Sanders. 1994. Expression of heterologous integrin genes in cultured eukaryotic cells. *Methods Enzymol.* 245:297–316.

Gipson, I.K., S. Spurr-Michaud, A. Tisdale, J. Elwell, and M.A. Stepp. 1993. Redistribution of the hemidesmosome components alpha 6 beta 4 integrin and bullous pemphigoid antigens during epithelial wound healing. *Exp. Cell Res.* 207:86–98.

Green, H. 1977. Terminal differentation of cultured human epidermal cells. *Cell.* 11:405–416.

Green, M.R., C. Mycock, C.G. Smith, and J.R. Couchman. 1987. Biochemical and ultrastructural processing of [^{125}I]-epidermal growth factor in rat epidermis and hair follicles: accumulation of nuclear label. *J. Invest. Dermatol.* 88: 259–265.

Guan, J.-L., and D. Shalloway. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature (Lond.).* 358:690–692.

Guo, L., L. Degestein, J. Dowling, Q-C. Yu, R. Wollmam, B. Perman, and E. Fuchs. 1995. Gene targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. *Cell.* 81:233–243.

Hall, P.A., and F.M. Watt. 1989. Stem cells: the generation and maintenance of cellular diversity. *Development* 106:619–633.

Hanks, S.K., M.B. Calalb, M.C. Harper, and S.K. Patel. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. USA.* 89:8487–8491.

Herman., B., and W.J. Pledger. 1985. Platelet-derived growth factor-induced alterations in vinculin and actin distribution in BALB/c-3T3 cells. *J. Cell Biol.* 100:1031–1040.

Honegger, A.M., T.J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger. 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell.* 50:199–209.

Hynes, R.O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. *Cell.* 69:11–25.

Izumi, K., Y. Hirao, L. Hopp, and R. Oyasu. 1981. In vitro induction of ornithine decarboxylase in urinary bladder carcinoma cells. *Cancer Res.* 41:405–409.

Juliano, R.L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.

Kahn-Perles, B., C. Boyer, B. Arnold, A.R. Sanderson, P. Ferrier, and F. Lemonnier. 1987. Acquisition of HLA class I W6/32 defined antigenic determinant by heavy chains from different species following association with bovine β_2-microglobulin. *J. Immunol.* 138:2190–2196.

Kajiji, S., R.N. Tamura, and V. Quaranta. 1989. A novel integrin ($\alpha_E\beta_4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:673–680.

Kamps, M.P., and B.M. Sefton. 1988. Identification of novel polypeptide substrates of the v-src, v-yes, v-fps, v-ros, v-erb-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene.* 2:305–315.

Kimmel, K.A., and T.E. Carey. 1986. Altered expression in squamous carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.* 46:3614–2623.

Kinashi, T., J.A. Escobedo, L.T. Williams, K. Takatsu, and T.A. Springer. 1995. Receptor tyrosine kinase stimulates cell-matrix adhesion by phosphatidylinositol 3 kinase and phospholipase C-γ1 pathways. *Blood.* 86:2086–2090.

Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin $\alpha_v\beta_5$-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.

Kurpakus, M.A., V. Quaranta, and J.C.R. Jones. 1991. Surface relocation of alpha$_6$ beta$_4$ integrins and assembly of hemidesmosomes in an in vitro model of wound healing. *J. Cell Biol.* 115:1737–1750.

Lee, E.C., M.M. Lotz, G.D. Steele, and A.M. Mercurio. 1992. The integrin $\alpha_6\beta_4$ is a laminin receptor. *J. Cell Biol.* 117: 671–678.

Lipfert, L., B. Haimovich, B.M. Shaller, B.S. Cobb, J.T. Parsons, and J.S. Brugge. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. *J. Cell Biol.* 119:905–912.

Mainiero, F., A. Pepe, K.K. Wary, L. Spinardi, M. Mohammadi, J. Schlessinger, and F.G. Giancotti. 1995. Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:4470–4481.

Martín-Padura, I., G. Bazzoni, A. Zanetti, S. Bernasconi, M.J. Elices, A. Mantovani, and E. Dejana. 1994. A novel mechanism of colon carcinoma cell adhesion to the endothelium triggered by β_1 integrin chain. *J. Biol. Chem.* 269: 6124–6132.

Matthay, M., J. Thiery, F. Lafont, M. Stampfer, and B. Boyer. 1993. Transient effect of epidermal growth factor on the motility of an immmortalized mammary epithelial cell line. *J. Cell Sci.* 106:869–878.

McNamee, H.M., D.E. Ingber, and M.A. Schwartz. 1992. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-inositol lipid breakdown. *J. Cell Biol.* 121:673–678.

Niessen, C.M., F. Hogervorst, L.H. Jaspars, A.A. De Melker, G.O. Delwel, E.H.M. Hulsman, I. Kuikman, and A. Sonnenberg. 1994. The $\alpha_6\beta_4$ integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.* 2111:360–367.

Nobes, C.D., and A. Hall. 1995. Rho, rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell.* 81:53–62.

Ozanne, B., C.S. Richards, F. Hendler, D. Burns, and B. Gusterson. 1986. Overexpression of the EGF receptor is a hallmark of squamous cell carcinoma. *J. Pathol.* 149:9–14.

Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I.

Nicoletti, F. Grignani, T. Pawson, and P.G. Pelicci. 1992. A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell.* 70:93–104.

Pellegrini, G., M. De Luca, G. Orecchia, F. Balzac, O. Cremona, P. Savoia, R. Cancedda, and P.C. Marchisio. 1992. Expression, topography, and function of integrin receptors are severely altered in keratinocytes from involved and uninvolved psoriatic skin. *J. Clin. Invest.* 89:1783–1795.

Rheinwald, J.G., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.).* 265: 421–424.

Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.

Schlaepfer, D.D., S.K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond.).* 372:786–791.

Schultz, G., D.S. Rotatori, and W. Clark. 1991. EGF and TGF-alpha in wound healing and repair. *J. Cell. Biochem.* 45:346–352.

Schwartz, M.A., M.D. Shaller, and M.H. Ginsberg. 1995. Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11:549–599.

Serve, H., N.S. Yee, G. Stella, L. Sepp-Lorenzino, J.C. Tan, and P. Besmer. 1995. Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cell. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:473-483.

Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.

Sonnenberg, A., C.J.T. Linders, J.H. Daams, and S.J. Kennel. 1990. The $\alpha_6\beta_1$ (VLA-6) and $\alpha_6\beta_4$ protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.* 96:207–217.

Sonnenberg, A., A.A. de Melker, A.M. Martinez de Velasco, H. Janssen, J. Calafat, and C.M. Niessen. 1993. Formation of hemidesmosomes in cells of a transformed murine cell line and mechanisms involved in adherence of these cells to laminin and kalinin. *J. Cell Sci.* 106:1083–1102.

Spinardi, L., Y.-L. Ren, R. Sanders, and F.G. Giancotti. 1993. The β_4 subunit cytoplasmic domain mediates the interaction of $\alpha_6\beta_4$ integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell.* 4:871–884.

Spinardi, L., S. Einheber, T. Cullen., T.A. Milner, and F.G. Giancotti. 1995. A recombinant tail-less integrin β_4 subunit disrupts hemidesmosomes, but does not suppress $\alpha_6\beta_4$-mediated cell adhesion to laminins. *J. Cell Biol.* 129:473–487.

Stepp, M.A., S. Spurr-Michaud, A. Tisdale, J. Elwell, and I.K. Gipson. 1990. Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl. Acad. Sci. USA.* 87:8970–8974.

Teslenko, L.V., E.S. Kornilova, A.D. Sorkin, and N.N. Nikolsky. 1987. Recycling of epidermal growth factor in A431 cells. *FEBS Lett.* 221:105–109.

Uitto, J., and A. Christiano. 1992. Molecular genetics of the cutaneous basement membrane zone. *J. Clin. Invest.* 90:687–692.

Ullrich, A., L. Coussens, J.S. Hayflick, T.J. Dull, A. Gray, A.W. Tam, J. Lee, Y. Yarden, T.A. Libermann, J. Schlessinger et al. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature (Lond.).* 309:418–425.

Vogel, B.E., S.J. Lee, A. Hildebrand, W. Craig, M. Pierschbacher, F. Wong-Staal, and E. Ruoslathi. 1993. A novel integrin specificity exemplified by binding of the $\alpha_v\beta_5$ integrin to the basic domain of the HIV tat protein and vitronectin. *J. Cell Biol.* 121:461–468.

Vuori, K., and E. Ruoslahti. 1994. Association of insulin receptor substrate-1 with integrins. *Science (Wash. DC).* 266:1576–1578.

Yamamoto, T., N. Kamata, H. Kawano, S. Shimizu, T. Kuroki, K. Toyoshima, K. Rikimaru, N. Nomura, R. Ishizaki, I. Pastan et al. 1986. High incidence of amplification of the epidermal growth factor gene in human squamous cell lines. *Cancer Res.* 46:414–416.

Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase (p125^{FAK}): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71:891–894.

The EMBO Journal Vol.16 No.9 pp.2365–2375, 1997

The coupling of $\alpha_6\beta_4$ integrin to Ras–MAP kinase pathways mediated by Shc controls keratinocyte proliferation

Fabrizio Mainiero[1,2], Chiara Murgia[1,3], Kishore K.Wary[1,3], Anna Maria Curatola[1,4], Angela Pepe[1,3], Miroslav Blumemberg[5], John K.Westwick[6], Channing J.Der[6] and Filippo G.Giancotti[1,3,7]

[1]Department of Pathology and [5]Department of Dermatology, Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016 and [6]Department of Pharmacology, Linenberg Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[2]Present address: Dipartimento di Medicina Sperimentale e Patologia, Università La Sapienza, Viale Regina Elena 324, 00161 Roma, Italy
[3]Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
[4]Present address: Department of Pediadrics, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA

[7]Corresponding author
e-mail: F-GIANCOTTI@ski.mskcc.org

The signaling pathways linking integrins to nuclear events are incompletely understood. We have examined intracellular signaling by the $\alpha_6\beta_4$ integrin, a laminin receptor expressed in basal keratinocytes and other cells. Ligation of $\alpha_6\beta_4$ in primary human keratinocytes caused tyrosine phosphorylation of Shc, recruitment of Grb2, activation of Ras and stimulation of the MAP kinases Erk and Jnk. In contrast, ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ did not cause these events. While the stimulation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative Shc, Ras and RhoA, the activation of Jnk was inhibited by dominant-negative Ras and Rac1 and by the phosphoinositide 3-kinase inhibitor Wortmannin. Adhesion mediated by $\alpha_6\beta_4$ induced transcription from the Fos serum response element and promoted cell cycle progression in response to mitogens. In contrast, $\alpha_3\beta_1$- and $\alpha_2\beta_1$-dependent adhesion did not induce these events. These findings suggest that the coupling of $\alpha_6\beta_4$ integrin to the control of cell cycle progression mediated by Shc regulates the proliferation of basal keratinocytes and possibly other cells which are in contact with the basement membrane *in vivo*.

Keywords: integrins/keratinocytes/MAP kinase/Ras/Shc

Introduction

In addition to promoting cell adhesion and contributing to the organization of tissues and organs, basement membranes exert complex and often divergent effects on the survival, proliferation and differentiation of epithelial cells (Adams and Watt, 1993; Lin and Bissel, 1993). The influences of basement membranes on epithelial cells are likely to be mediated by the ability of laminin-binding integrins to induce intracellular signaling (Giancotti and Mainiero, 1994; Clark and Brugge, 1995; Schwartz *et al.*, 1995), but the mechanisms involved are incompletely understood.

To elucidate the effects of laminins on epithelial cells, we have focused on the $\alpha_6\beta_4$ integrin. Cell adhesion assays with $\alpha_6\beta_4$-transfected K562 cells and radioligand binding studies with purified recombinant $\alpha_6\beta_4$ have indicated that this integrin is a receptor for various laminin isoforms and binds with the highest apparent affinity to laminins 5 and 4 (Niessen *et al.*, 1994; Spinardi *et al.*, 1995). In accordance with its role as a basement membrane receptor, $\alpha_6\beta_4$ is expressed in epithelial cells (Kajiji *et al.*, 1989), Schwann cells (Sonnenberg *et al.*, 1990; Einheber *et al.*, 1993) and a subset of endothelial cells (Kennel *et al.*, 1992; Klein *et al.*, 1993) and thymocytes (Wadsworth *et al.*, 1992).

The $\alpha_6\beta_4$ integrin has a distinctive structure and subcellular localization. The large cytoplasmic domain of the β_4 subunit, which is characterized by two pairs of type III fibronectin (Fn)-like domains separated by a 142 amino acid sequence (connecting segment), does not contain any region of homology with the cytoplasmic domains of other known integrin β subunits (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Furthermore, while β_1 and α_v integrins are concentrated in focal adhesions and linked to the actin filament system, the $\alpha_6\beta_4$ integrin is found in hemidesmosomes both *in vivo* and in cultured cells (Carter *et al.*, 1990a; Stepp *et al.*, 1990). These observations suggest that the unique cytoplasmic domain of β_4 interacts with cytoskeletal elements of hemidesmosomes, thereby linking $\alpha_6\beta_4$ to the keratin filament system.

Our previous studies have indicated that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton is mediated by the cytoplasmic domain of β_4 and specifically by a region which includes the first pair of type III Fn-like repeats and the connecting segment (Spinardi *et al.*, 1993). In accordance with the hypothesis that $\alpha_6\beta_4$ plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system, we have observed that the introduction of a truncated tail-less β_4 subunit into cells possessing endogenous $\alpha_6\beta_4$ integrins and hemidesmosomes results in a dominant-negative effect on hemidesmosome assembly (Spinardi *et al.*, 1995). Since the tail-less integrin binds efficiently to extracellular ligand, its dominant-negative effect is likely to result from its ability to co-cluster with the endogenous wild-type receptor and block a signal necessary for hemidesmosome assembly. In accordance with this hypothesis, recent studies have revealed that $\alpha_6\beta_4$ is associated with an intracellular tyrosine kinase. Mutagenesis experiments have provided evidence that the phosphorylation of a tyrosine activation motif (TAM) located in the connecting segment controls the association of $\alpha_6\beta_4$ with the hemidesmosomal

cytoskeleton, presumably via the recruitment of a signaling molecule containing two tandem Src homology 2 (SH2) domains (Mainiero *et al.*, 1995).

In the epidermis and other stratified epithelia, the expression of $\alpha_6\beta_4$ is restricted to the basal cell layer which contains cells endowed with proliferative capacity (Kajiji *et al.*, 1989). It is known that keratinocytes exit the cell cycle and begin their differentiation program when they detach from the basement membrane to migrate to the upper epidermal layers (Hall and Watt, 1989). In fact, this process can be replicated *in vitro* by depriving cultured keratinocytes of anchorage to their endogenously produced extracellular matrix (Green, 1977), which is particularly rich in laminin 5 (Carter *et al.*, 1991; Rousselle *et al.*, 1991). Furthermore, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of $\alpha_6\beta_4$ (Kimmel and Carey, 1986; Wolf *et al.*, 1990). Finally, the basal keratinocytes of β_4 knock-out mice display signs of degeneration even in areas of epidermis where no significant detachment from the basement membrane is observed (Dowling *et al.*, 1996). These observations suggest that $\alpha_6\beta_4$ may provide epithelial cells with a signal important for cell survival and cell cycle progression.

What is the mechanism by which the $\alpha_6\beta_4$ integrin transduces biochemical signals capable of affecting cell proliferation? Immunoprecipitation and GST fusion protein binding experiments have indicated that ligation of $\alpha_6\beta_4$ results in the association of the adaptor protein Shc with tyrosine-phosphorylated β_4. Shc is then phosphorylated on tyrosine residues, presumably by the integrin-associated kinase, and combines with the other adaptor protein Grb2 (Mainiero *et al.*, 1995). These observations raise two key questions. First, what are the biochemical consequences of the recruitment of Shc and Grb2 to $\alpha_6\beta_4$? Second, what is the biological significance of $\alpha_6\beta_4$ signaling in epithelial cells?

In this study, we provide evidence that the $\alpha_6\beta_4$ integrin stimulates the Ras–Erk and Rac–Jnk mitogen-activated protein kinase (MAP kinase) signaling pathways via Shc and thereby controls immediate-early gene expression and keratinocyte proliferation in response to laminin.

Results

Ligation of $\alpha_6\beta_4$ causes activation of the Ras–Erk signaling pathway

To examine the intracellular signaling pathways activated by the $\alpha_6\beta_4$ integrin in a physiologically relevant cellular context, we elected to use primary human keratinocytes. These cells express high levels of $\alpha_6\beta_4$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ and adhere to laminin 5 through $\alpha_6\beta_4$ and $\alpha_3\beta_1$ (Xia *et al.*, 1996) and to collagen I through $\alpha_2\beta_1$ (Carter *et al.*, 1990b). Incubation of suspended keratinocytes with polystyrene beads coated with the anti-β_4 monoclonal antibody (Mab) 3E1 as well as adhesion to laminin 5-coated dishes caused tyrosine phosphorylation of the 52 and 46 kDa mol. wt isoforms of Shc (the 66 kDa isoform of Shc is expressed at very low levels in keratinocytes) and thereby recruitment of Grb2 (Figure 1A). No significant tyrosine phosphorylation of Shc and recruitment of Grb2 was observed in keratinocytes treated with beads coated with the anti-$\alpha_3\beta_1$ Mab P1B5 (not shown) or the control anti-MHC Mab W6.32 and in keratinocytes adhering to collagen I or poly-L-lysine-coated dishes (Figure 1A). The inability of collagen I to induce tyrosine phosphorylation of Shc and recruitment of Grb2 was not a consequence of insufficient adhesion because the keratinocytes spread equally well on laminin 5 and collagen I under our experimental conditions. Furthermore, we observed that antibody-mediated cross-linking of $\alpha_2\beta_1$ does not induce tyrosine phosphorylation of Shc and recruitment of Grb2 (not shown). These findings, which are consistent with previous results (Mainiero *et al.*, 1995; Wary *et al.*, 1996), indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_2\beta_1$ and $\alpha_3\beta_1$, can promote signaling mediated by Shc in primary keratinocytes.

We next examined the role of cytoplasmic domain of β_4 in the recruitment and tyrosine phosphorylation of Shc. Rat bladder 804G cells expressing either a recombinant full-length human β_4 subunit (clone A) or a tail-less version (clone L) were cross-linked in suspension with the anti-β_4 Mab 3E1 or the control anti-MHC Mab W6.32. Immunoprecipitation with the 3E1 Mab followed by immunoblotting with anti-Shc antibodies revealed that the recruitment of all three isoforms of Shc by $\alpha_6\beta_4$ requires the cytoplasmic domain of the β_4 subunit (Figure 1B). To examine if recruitment to $\alpha_6\beta_4$ was a prerequisite for tyrosine phosphorylation of Shc, clone A and clone L were either cross-linked in suspension with the 3E1 or the W6.32 Mab or plated onto dishes coated with the same antibodies. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-P-Tyr antibodies indicated that ligation of wild-type $\alpha_6\beta_4$ induces tyrosine phosphorylation of the 52 kDa isoform of Shc (the major isoform recruited to activated $\alpha_6\beta_4$ in these cells). In contrast, ligation of tail-less $\alpha_6\beta_4$ did not cause efficient tyrosine phosphorylation of Shc (Figure 1C). These results are consistent with the hypothesis that the recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by tyrosine phosphorylation of the cytoplasmic domain of β_4. They also suggest that this event is necessary for subsequent tyrosine phosphorylation of the adaptor protein. Since in a number of experiments tail-less $\alpha_6\beta_4$ was able to induce a modest level of tyrosine phosphorylation of Shc, it is possible that an additional indirect mechanism contributes to the activation of Shc by $\alpha_6\beta_4$.

Since Grb2 is stably associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane mediated by Shc is likely to bring mSOS in close proximity to its target Ras (Schlessinger, 1994). Ras-GTP loading experiments were therefore performed to examine if ligation of $\alpha_6\beta_4$ resulted in activation of Ras. After growth factor starvation and *in vivo* labeling with [^{32}P]orthophosphate, primary human keratinocytes were detached and either incubated in suspension with anti-β_4 or anti-MHC beads or replated on dishes coated with laminin 5 or anti-β_4 Mab. As a control, adherent keratinocytes were either left untreated or were stimulated with epidermal growth factor (EGF). As shown in Figure 2, chromatographic analysis of nucleotides bound to Ras indicated that adhesion to laminin 5- or anti-β_4 Mab-coated dishes results in an accumulation of GTP on Ras comparable with that caused by EGF. In suspended cells, however, antibody-mediated ligation of $\alpha_6\beta_4$ did not cause activation of Ras (Figure 2). The results of this experiment indicate that $\alpha_6\beta_4$-mediated cell adhesion causes activation

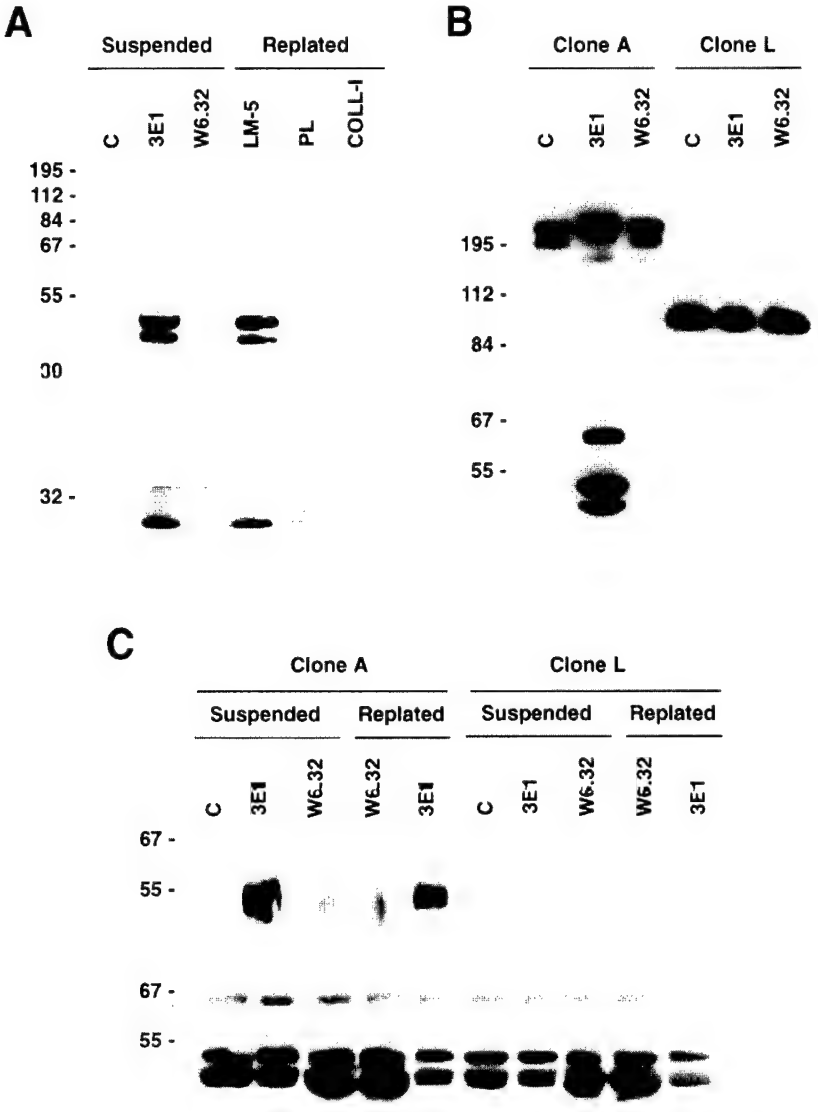


Fig. 1. Ligation of the $\alpha_6\beta_4$ integrin induces recruitment of Shc, tyrosine phoshorylation of Shc and association of Shc with Grb2. (**A**) Tyrosine phoshorylation of Shc and association of Shc with Grb2 in response to $\alpha_6\beta_4$ ligation. After growth factor starvation, primary human keratinocytes were detached and either incubated in suspension with medium alone (C), anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Grb2 antibodies (bottom panel). (**B**) The recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4. Clone A and clone L cells were serum starved and incubated in suspension with medium alone (C), with anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min. Equal amounts of total proteins were immunoprecipitated with 3E1 Mab. The samples were probed by immunoblotting with polyclonal antibodies to the ectodomain of β_4 (top panel) and to Shc (bottom panel). (**C**) The cytoplasmic domain of β_4 is important for the activation of Shc in response to $\alpha_6\beta_4$ ligation. Clone A and clone L cells were serum starved and either incubated in suspension with medium alone (C), anti-β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with anti-β_4 (3E1) or anti-MHC (W6.32) Mab for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Shc antibodies (bottom panel).

of Ras. They also suggest that, in contrast to the sequential recruitment of Shc and Grb2 which can occur in suspended cells treated with anti-β_4 beads, optimal activation of Ras by $\alpha_6\beta_4$ requires adhesion and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands.

We next examined if ligation of $\alpha_6\beta_4$ resulted in activation of the MAP kinase Erk. Growth factor-starved keratinocytes were detached and either kept in suspension or plated on dishes coated with the anti-β_4 Mab 3E1, the anti-MHC Mab W6.32, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were treated with EGF. As shown in Figure 3A, immunoprecipitation and *in vitro* kinase assays indicated that adhesion to laminin 5- or anti-β_4 Mab-coated dishes causes activation of Erk to a level similar to that induced by EGF. In contrast, adhesion to collagen I, poly-L-lysine or anti-MHC Mab did not result in significant activation of Erk (Figure 3A). Adhesion to anti-α_3 Mab-coated dishes also did not cause Erk activation (not shown). To examine the kinetics of Erk activation by $\alpha_6\beta_4$, keratinocytes were plated on laminin 5 for various times and subjected to Erk immunoprecipitation and kinase assay. As shown in Figure 3B, we detected a significant activation of Erk as early as 5 min after plating the keratinocytes on laminin

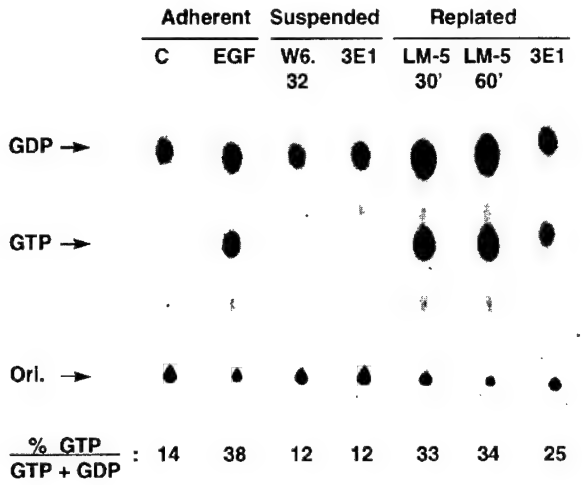


Fig. 2. Activation of Ras by the $\alpha_6\beta_4$ integrin. Primary human keratinocytes were growth factor starved and labeled *in vivo* with [^{32}P]orthophosphate. After detachment, they were then either incubated in suspension with anti-MHC (W6.32) or anti-β_4 (3E1) Mab-coated beads for 20 min or plated on dishes coated with laminin 5 (LM-5) for the indicated times or on dishes coated with anti-β_4 Mab (3E1) for 30 min. As a control, adherent cells were either left untreated or stimulated with 200 ng/ml EGF for 5 min. After immunoprecipitation of Ras, bound nucleotides were eluted and separated by TLC. Numbers indicate the molar ratio of GTP over total nucleotides.

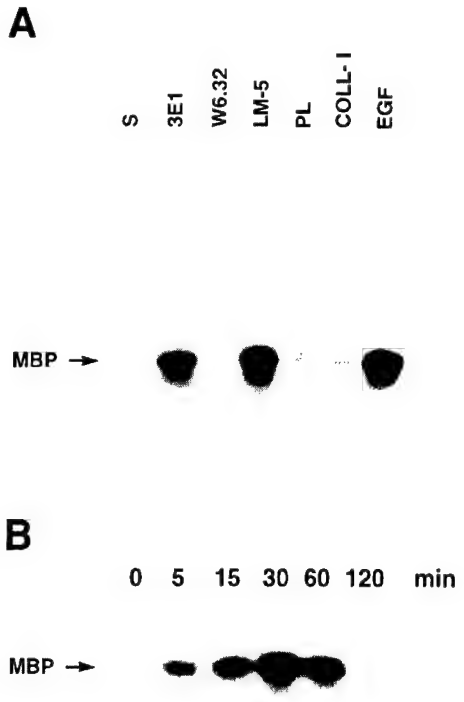


Fig. 3. Activation of Erk kinase by $\alpha_6\beta_4$. (**A**) Specificity of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), collagen I (COLL-I) or poly-L-lysine (PL) for 30 min. As a control, adherent cells were stimulated with 200 ng/ml EGF for 5 min (EGF). (**B**) Kinetics of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension or plated on dishes coated with laminin 5 for the indicated times. Anti-Erk immunoprecipitates were subjected to *in vitro* kinase assay using MBP as a substrate.

5. The level of Erk activity peaked at 30 min of adhesion and declined thereafter. These results indicate that ligation of $\alpha_6\beta_4$ causes a significant and relatively persistent activation of the MAP kinase Erk and are in agreement with the observation that ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ does not induce this event (Wary *et al.*, 1996). Since adhesion mediated by $\alpha_3\beta_1$ and $\alpha_2\beta_1$ is known to cause activation of focal adhesion kinase (FAK), these findings are also consistent with the notion that activation of FAK is not sufficient for stimulation of Erk in response to integrin ligation (Wary *et al.*, 1996).

The activation of Erk mediated by $\alpha_6\beta_4$ requires Shc, Ras and Rho

The mechanism of Erk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins. Since transient transfection of primary keratinocytes is very inefficient, we elected to use HeLa cells which express levels of $\alpha_6\beta_4$, $\alpha_3\beta_1$ and $\alpha_2\beta_1$ comparable with those of primary keratinocytes. HeLa cells were transfected with a hemagglutinin (HA)-tagged Erk2 vector in combination with different concentrations of cDNAs encoding dominant-negative Shc (317F), Ras (N17), RhoA (N19), CDC42 (N17) and Rac1 (N17). As shown in Figure 4, immunoprecipitation of HA-Erk2 followed by *in vitro* kinase assay indicated that the activation of Erk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Shc (Figure 4A), Ras and RhoA (Figure 4B), but not by dominant-negative Cdc42 and Rac1 (Figure 4C). Although at the highest concentration tested dominant-negative Shc, Ras and RhoA completely inhibited the activation of Erk by $\alpha_6\beta_4$, at the intermediate and lowest concentration tested the inhibitory activity of the three dominant-negative mutants differed, perhaps as a consequence of the different relative abundance of their target protein or their different mechanism of action. These results indicate that Shc couples the $\alpha_6\beta_4$ integrin to the Ras–Erk signaling pathway and suggest that full activation of Erk in response to $\alpha_6\beta_4$ ligation requires the activity of both Ras and Rho.

Ligation of $\alpha_6\beta_4$ causes activation of the Rac–Jnk signaling pathway

We next examined if ligation of $\alpha_6\beta_4$ stimulated the MAP kinase Jnk. Growth factor-starved primary keratinocytes were detached and either kept in suspension or plated on dishes coated with anti-β_4 Mab, anti-MHC Mab, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were exposed to UV light. Jnk was precipitated by using a GST–Jun fusion protein and its activity examined by *in vitro* kinase assay. As shown in Figure 5, the binding of Mab 3E1 or laminin 5 to $\alpha_6\beta_4$ caused an activation of Jnk comparable with that induced by UV stimulation. In contrast, adhesion to dishes coated with collagen I, poly-L-lysine, the control Mab W6.32 (Figure 5) or the anti-$\alpha_3\beta_1$ Mab P1B5 (not shown) did not result in significant activation of Jnk. Time-course experiments indicated that the kinetics of activation of Jnk in response to laminin 5 were similar to those observed for Erk (not shown). These results indicate that ligation of $\alpha_6\beta_4$ causes activation of Jnk, and suggest that ligation of the collagen-binding integrin $\alpha_2\beta_1$ does not induce this event.

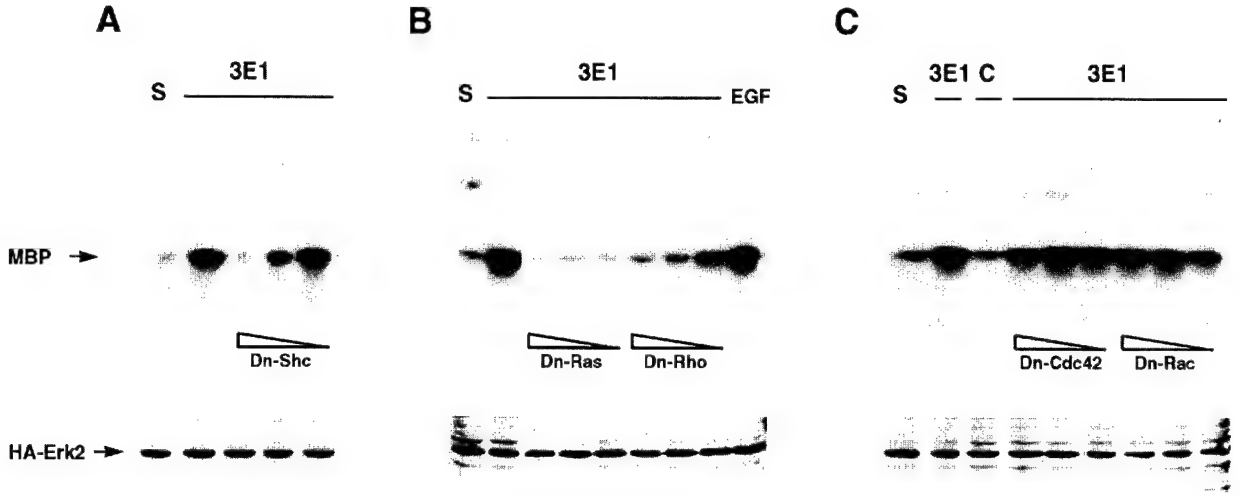


Fig. 4. The activation of Erk caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative Shc, Ras and Rho. (**A**) HeLa cells were transiently transfected with 3 μg of HA-tagged Erk2 plasmid alone or in combination with 10, 5 and 2.5 μg of vector encoding dominant-negative Shc (Dn-Shc). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) for 30 min. (**B**) HeLa cells were transiently transfected with 1 μg of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Ras (Dn-Ras) or RhoA (Dn-Rho). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) for 30 min. As a control, adherent cells were treated with 200 ng/ml EGF for 5 min. (**C**) HeLa cells were transiently transfected with 1 μg of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Cdc42 (Dn-Cdc42) or Rac (Dn-Rac). The cells were then either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1) or anti-MHC Mab (C) for 30 min. Anti-HA immunoprecipitates were subjected to *in vitro* kinase assay using myelin basic protein (MBP) as a substrate (top panels). Transfection efficiences were verified by immunoblotting aliquots of total proteins with anti-HA antibodies (bottom panels).

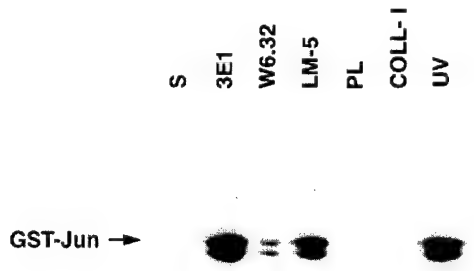


Fig. 5. Activation of Jnk kinase by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti-β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 20 min. As a control, adherent cells were exposed to 40 J/m^2 of UV radiation (UV) and then kept in culture for 20 min. Jnk kinase was precipitated using glutathione beads coated with GST–Jun fusion protein and subjected to *in vitro* kinase assay. The position of phosphorylated GST–Jun is indicated. The lower band is a degradation product of GST–Jun.

The activation of Jnk mediated by $\alpha_6\beta_4$ requires Ras, PI-3K and Rac

The mechanism of Jnk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins and the phosphoinositide 3-kinase (PI-3K) inhibitor Wortmannin. HeLa cells were transfected with a Flag-tagged Jnk1 vector in combination with different concentrations of cDNAs encoding dominant-negative Ras (N17), Rac1 (N17), Cdc42 (N17) and RhoA (N19). As shown in Figure 6A, precipitation of Flag-Jnk1 followed by *in vitro* kinase assay indicated that the activation of Jnk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Ras and Rac1, but not by dominant-negative RhoA, and very modestly by dominant-negative Cdc42. These results indicate that the activation of Jnk by $\alpha_6\beta_4$ requires the activity of both Ras and Rac. Although it has been suggested that Raf can activate Jnk by acting on the MAP kinase kinase kinase MEKK1 (Lange-Carter and Johnson, 1994), recent results indicate that the predominant mechanism by which Ras activates Jnk involves Rac, and not Raf (Kosravi-Far *et al.*, 1995; Minden *et al.*, 1995; Qiu *et al.*, 1995; Joneson *et al.*, 1996). The requirement for Rac in our system is consistent with this hypothesis. Since PI-3K is a downstream target effector of Ras (Rodriguez-Viciana *et al.*, 1994) and has been implicated in the activation of Rac (Nobes *et al.*, 1995; Klippel *et al.*, 1996), we tested if inhibition of PI-3K interfered with the activation of Jnk by $\alpha_6\beta_4$. As shown in Figure 6B, the activation of Jnk in response to ligation of $\alpha_6\beta_4$ was completely suppressed by as little as 50 nM Wortmannin, a concentration at which the inhibitor has very little effect on signaling molecules other than PI-3-K (Wymann *et al.*, 1996). Taken together, the results of these experiments suggest that $\alpha_6\beta_4$ activates the Rac–Jnk signaling pathway via Ras and PI-3K.

Induction of Fos SRE-dependent transcription by $\alpha_6\beta_4$

We next examined if the coupling of $\alpha_6\beta_4$ to Shc played a role in the control of immediate-early gene expression. Since Erk regulates transcription from the Fos serum response element (SRE) by phosphorylating the ternary complex factors Elk-1 and SAP-1, and Rho family proteins cooperate with this Erk function by acting on the serum response factor (Treisman, 1995), we examined the effect of $\alpha_6\beta_4$ ligation on the Fos SRE. HeLa cells were

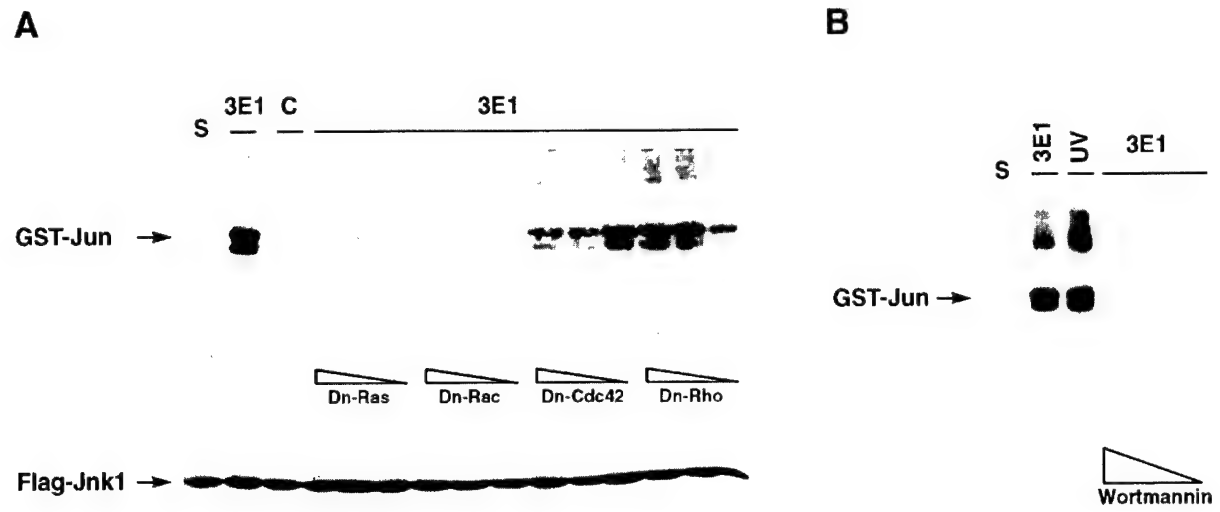


Fig. 6. The activation of Jnk kinase caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative versions of Ras and Rac and by the PI-3K inhibitor Wortmannin. (**A**) HeLa cells were transiently transfected with 1 μg of Flag-tagged Jnk1 plasmid alone or in combination with 1, 0.5 and 0.25 μg of vectors encoding dominant-negative Ras (Dn-Ras), Rac (Dn-Rac), Cdc42 (Dn-Cdc42) and RhoA (Dn-Rho). The cells were then either kept in suspension or plated on dishes coated with anti-β_4 Mab (3E1) or anti-MHC Mab (C) for 20 min. Flag-Jnk was immunoprecipitated with the anti-Flag Mab M2 and subjected to *in vitro* kinase assay with GST–Jun as a substrate (top panel). Transfection efficiences were verified by immunoblotting aliquots of total proteins with Mab M2 (bottom panel). (**B**) HeLa cells were transiently transfected with 1 μg of Flag-tagged Jnk1 plasmid. After detachment, the cells were either kept in suspension or plated on dishes coated with anti-β_4 Mab (3E1) in the absence or presence of 200, 100 or 50 nM Wortmannin for 20 min. As a control, adherent cells were exposed to 40 J/m^2 of UV radiation for 20 min. Flag-Jnk was immunoprecipitated with Mab M2 and subjected to *in vitro* kinase assay with GST–Jun as a substrate. The position of phosphorylated GST–Jun is indicated. The lower band is a degradation product of GST–Jun.

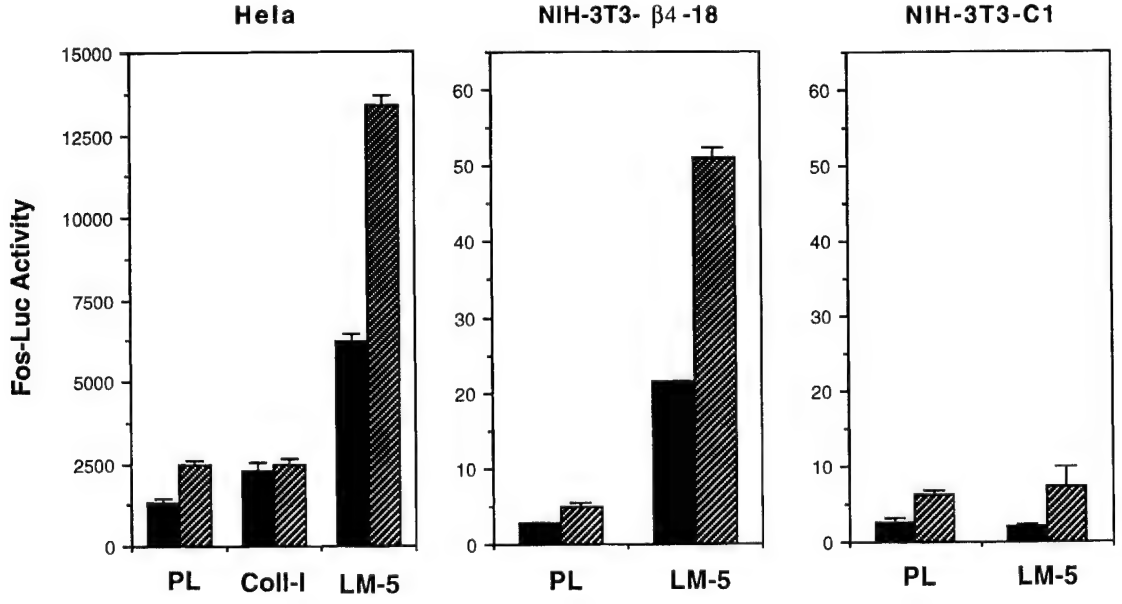


Fig. 7. Adhesion mediated by $\alpha_6\beta_4$ promotes transcription from the Fos SRE. HeLa cells, β_4-expressing NIH 3T3-β_4-18 cells and control NIH 3T3-C1 cells were transiently transfected with Fos-SRE–Luc plasmid. After growth factor starvation, the cells were detached and plated onto dishes coated with 10 μg/ml poly-L-lysine (PL), laminin 5 (LM-5) or collagen I (Coll-I) for 30 min. The cells were then either left untreated (solid bars) or exposed to mitogens for 10 min (shaded bars). Cell lysates were subjected to luciferase assay. Values are expressed in arbitrary units. The diagram shows the mean value and standard deviation from triplicate samples.

transiently transfected with the Fos-SRE–Luc vector, which contains the Fos SRE promoter element linked to the luciferase reporter gene. Upon plating on dishes coated with poly-L-lysine, collagen I or laminin 5, the cells were either left untreated or exposed to EGF. They were then subjected to luciferase assay. As shown in Figure 7, while adhesion to laminin 5 in the absence of EGF caused elevation of Fos SRE-dependent transcription, adhesion to poly-L-lysine or collagen I under the same conditions did not induce this activity. This suggests that ligation of $\alpha_6\beta_4$, but not $\alpha_2\beta_1$, is sufficient to promote transcription from the Fos SRE in the absence of mitogens. Treatment with EGF induced a significant elevation of Fos SRE activity in HeLa cells adhering to laminin 5, but caused a remarkably modest effect in cells attaching to poly-L-lysine or collagen I. This result suggests that ligation of $\alpha_6\beta_4$ is required for optimal induction of Fos SRE-dependent transcription in response to EGF.

To examine if laminin 5 is able to induce transcription from the Fos SRE in normal untransformed cells and to demonstrate the role of $\alpha_6\beta_4$ in this process, we transfected NIH 3T3 fibroblasts with a retroviral vector encoding human β_4 and isolated stable cell lines. Immunoprecipitation and fluorescence-activated cell sorting (FACS) analysis indicated that the recombinant β_4 subunit associated with endogenous α_6 and was regularly exported to the cell surface. Clones 8 and 18 displayed the highest levels of recombinant β_4 on the cell surface and were examined further. FACS analysis indicated that the level of expression of recombinant β_4 in these two clones approximated 40% of that of endogenous β_4 in primary keratinocytes. Since the NIH 3T3 cells do not express $\alpha_6\beta_4$ and adhere to laminin 5 through $\alpha_3\beta_1$, we examined the function of recombinant $\alpha_6\beta_4$ by comparing the kinetics by which the β_4 transfectants and control cells adhered to laminin 5-coated dishes. The results showed that the β_4 transfectants adhered to laminin 5 with faster kinetics than the controls. However, both types of cells became equally spread by 30 min of plating (not shown).

To analyze Fos SRE-dependent transcription in reponse to laminin 5, control and β_4-transfected NIH 3T3 cells were transiently transfected with the Fos-SRE–Luc plasmid. Upon plating on dishes coated with poly-L-lysine or laminin 5, the cells were either left untreated or exposed to basic fibroblast growth factor (bFGF), insulin and platelet-derived growth factor (PDGF). The results of luciferase assays indicated that adhesion to laminin 5 causes elevation of Fos SRE-dependent transcription in the β_4 transfectants even in the absence of mitogens and this induction is potentiated by mitogen treatment (Figure 7). The ability of laminin 5 to induce Fos SRE-dependent transcription was dependent on $\alpha_6\beta_4$ expression, because adhesion to laminin 5 did not cause this effect in control cells even after mitogen treatment (Figure 7). These results indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_3\beta_1$, is sufficient to induce Fos SRE-dependent transcription, and confirm that $\alpha_6\beta_4$ cooperates with mitogens to cause optimal induction of this activity.

Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression

To examine if $\alpha_6\beta_4$ signaling played a role in cell cycle progression, primary keratinocytes were growth factor starved and then plated in the presence of EGF on plastic wells coated with laminin 5, collagen I or poly-L-lysine. Entry into the S phase was examined by 5′-bromo-2′-deoxyuridine (BrdU) incorporation and anti-BrdU staining. As shown in Figure 8, a significant fraction of keratinocytes plated on laminin 5 entered into the S phase during the 22 h of the assay. In contrast, only a modest percentage of cells plated on collagen I or poly-L-lysine entered into S during the assay. In the absence of mitogens, a similarly small percentage of cells plated on collagen I or poly-L-lysine entered into S phase (not shown). This fraction may consist of unsynchronized cells, which have already passed the G_1–S boundary at the time of plating. In addition, because the keratinocytes acquired and maintained a well-spread morphology on collagen I, their inability to enter into S on this substratum is not the result of insufficient spreading. The results of these experiments indicate that physical attachment and spreading on the extracellular matrix is not sufficient for progression of keratinocytes through G_1 in response to EGF, and suggest that this process requires ligation of a specific integrin, such as $\alpha_6\beta_4$.

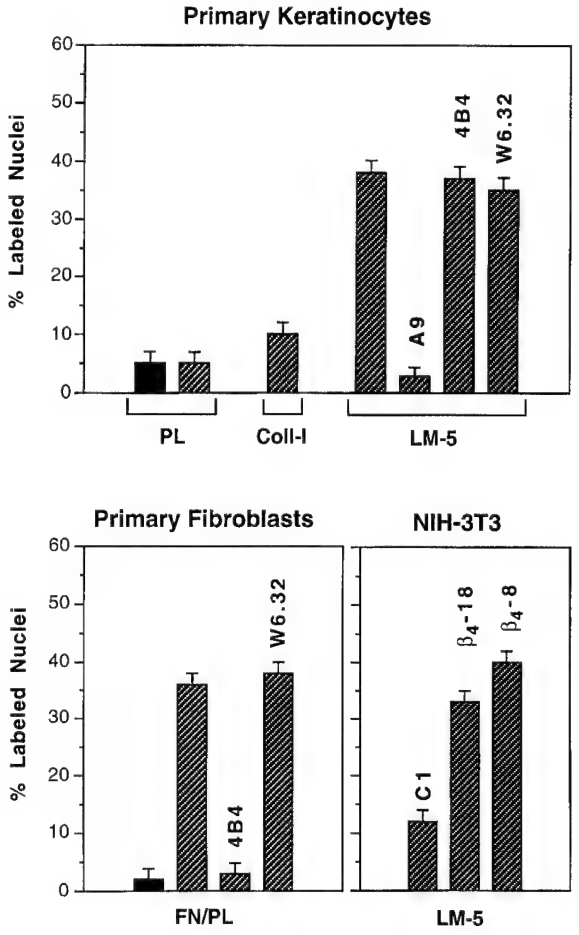


Fig. 8. Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression. Primary human keratinocytes, primary human dermal fibroblasts, control NIH 3T3-C1 cells and β_4-expressing NIH 3T3-18 and -8 cells were growth factor starved and plated on wells coated with 10 μg/ml poly-L-lysine (PL), collagen I (Coll-I) or laminin 5 (LM-5), or a mixture of 10 μg/ml fibronectin and 10 μg/ml poly-L-lysine (FN/PL). The cells were then incubated for 22 h in defined medium containing 10 μM BrdU and mitogens (shaded bars). To estimate the percentage of unsynchronized primary cells, the cells were also incubated for 22 h in defined medium containing 10 μM BrdU without mitogens (solid bars). When indicated, 10 μg/ml anti-β_1 Mab 4B4, 1:20 anti-β_4 Mab A9 ascites or 10 μg/ml control anti-MHC Mab W6.32 were included in the medium. After immunostaining with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies, the percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields. The diagram shows the mean value and standard deviation from triplicate samples.

To examine the relative roles of $\alpha_6\beta_4$ and $\alpha_3\beta_1$ in keratinocyte proliferation, we tested the effect of inhibitory anti-β_4 and anti-β_1 antibodies. Growth factor-starved keratinocytes were plated on laminin 5 and exposed to EGF in the presence of the inhibitory anti-β_1 Mab 4B4, the inhibitory anti-β_4 Mab A9 or the control anti-MHC Mab W6.32. As shown in Figure 8, exposure to the anti-β_4 Mab completely suppressed keratinocyte entry into S. In contrast, treatment with the anti-β_1 or anti-MHC Mab did not inhibit keratinocyte proliferation on laminin 5. To control the efficacy of the anti-β_1 Mab 4B4, G_0 syncronized

primary human fibroblasts were plated on a mixed substrate consisting of poly-L-lysine and fibronectin and exposed to mitogens in the presence of the 4B4 or W6.32 Mab. In accordance with the recent observation that a class of β_1 integrins, which include the $\alpha_5\beta_1$ fibronectin receptor, is linked to the Ras–Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996), plating of the primary fibroblasts on fibronectin/poly-L-lysine promoted cell cycle progression, and exposure to anti-β_1 Mab 4B4 blocked this process without inducing detachment (Figure 8). These results suggest that the ability of laminin 5 to promote keratinocyte cell cycle progression is mediated by $\alpha_6\beta_4$, and not by $\alpha_3\beta_1$.

We next examined the ability of control and β_4-expressing NIH 3T3 cells to progress through G_1 on laminin 5. While only a modest percentage of control cells progressed through G_1 when plated on laminin 5 for 22 h, a significant fraction of β_4 expressors entered into S under the same conditions (Figure 8), suggesting that ligation of $\alpha_6\beta_4$ is sufficient to promote progression through G_1 in response to mitogens. Taken together, the results of these assays indicate that ligation of $\alpha_6\beta_4$ is required and sufficient to promote keratinocyte proliferation in response to laminin 5.

Discussion

Although the notion that cell adhesion to the extracellular matrix regulates gene expression is supported by considerable experimental evidence, the signaling pathways linking integrins to nuclear events are not well known. In particular, the mechanisms by which integrin-dependent signals regulate cell cycle progression in normal epithelial cells are not fully understood. The results of recent studies have defined the membrane-proximal events induced by ligation of the $\alpha_6\beta_4$ integrin, a laminin receptor involved in various morphogenetic processes (Giancotti, 1996). Upon binding to extracellular ligand, $\alpha_6\beta_4$ becomes phosphorylated on tyrosine residues by the action of an integrin-associated kinase and thereby combines sequentially with the adaptor proteins Shc and Grb2 (Mainiero *et al.*, 1995). The results of the present study provide clear evidence that these receptor-proximal events result in the activation of Ras and of two distinct MAP kinase signaling pathways which regulate immediate-early gene expression. In contrast, other integrins, such as the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ collagen and laminin receptors, do not induce these events. Since $\alpha_6\beta_4$-mediated adhesion promotes keratinocyte progression through G_1 in response to growth factor treatment, while $\alpha_2\beta_1$- and $\alpha_3\beta_1$-mediated adhesion does not, we propose that the linkage of $\alpha_6\beta_4$ to Ras signaling mediated by Shc participates in the control of cell cycle progression in normal epithelial cells.

The adaptor protein Shc contains two separate domains involved in the recognition of tyrosine-phosphorylated sequence motifs: an N-terminal phosphotyrosine-binding (PTB) domain and a C-terminal Src homology 2 (SH2) domain (Pawson, 1995). GST fusion protein binding experiments have suggested that both domains can interact with the tyrosine-phosphorylated β_4 tail (Mainiero *et al.*, 1995). The results of this study are in accordance with the notion that the recruitment of Shc to $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4 and suggest that this event is important for the subsequent tyrosine phosporylation of Shc, which is presumably mediated by the integrin-associated kinase. Upon phosphorylation, Shc combines with the other adaptor protein Grb2. Since Grb2 is constitutively associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane potentially links $\alpha_6\beta_4$ to Ras. In accordance with this hypothesis, our current results show that ligation of $\alpha_6\beta_4$ results in a significant activation of Ras.

In contrast to the recruitment of Shc and Grb2 which could be observed in suspended keratinocytes cross-linked with anti-$\alpha_6\beta_4$ antibodies, full activation of Ras required physical attachment and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands. Since it has been suggested that proper targeting of the Grb2–mSOS complex to Ras may require an interaction of the Grb2 SH3 domains with the cortical cytoskeleton (Bar-Sagi *et al.*, 1993), it is possible that such targeting is defective in suspended keratinocytes cross-linked with anti-$\alpha_6\beta_4$ antibodies. Alternatively, since the pleckstrin homology domain of mSOS may bind to phosphatidylinositol(4,5)bisphosphate ($PtdInsP_2$) in the plasma membrane (Lemmon *et al.*, 1996) and it is known that $PtdInsP_2$ levels decline in suspended cells (McNamee *et al.*, 1993), it is possible that Ras activation by $\alpha_6\beta_4$ requires a threshold concentration of $PtdInsP_2$ in the plasma membrane which is not available in suspended keratinocytes. Future studies will be required to resolve this issue.

The results of this study indicate that ligation of $\alpha_6\beta_4$ results in the stimulation of both Ras–Erk and Rac–Jnk MAP kinase signaling pathways. The activation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative versions of both Shc and Ras, indicating that the coupling to Ras mediated by Shc is the major mechanism by which $\alpha_6\beta_4$ controls Erk activation. Interestingly, Erk activation was also inhibited by dominant-negative RhoA. This result, which is in agreement with the recent observation that Rho activity is required for full activation of Erk in response to various extracellular stimuli (Hill *et al.*, 1995), suggests that this G protein also participates in signaling by $\alpha_6\beta_4$. The activation of Jnk by $\alpha_6\beta_4$ was inhibited by dominant-negative Ras and Rac1, but not by dominant-negative RhoA and Cdc42. In addition, it was suppressed by nanomolar concentrations of the PI-3K inhibitor Wortmannin. Since there is evidence that PI-3K is a downstream target effector of Ras and is involved in the activation of Rac (Rodriguez-Viciana *et al.*, 1994; Nobes *et al.*, 1995; Klippel *et al.*, 1996), it is likely that $\alpha_6\beta_4$ stimulates the Rac–Jnk pathway via Ras. Thus, the coupling of $\alpha_6\beta_4$ to Ras mediated by Shc leads to the activation of both Ras–Erk and Rac–Jnk signaling pathways.

In accordance with the observation that Erk stimulates transcription of the immediate-early gene *fos* (Treisman, 1995), the results of our study indicate that adhesion mediated by $\alpha_6\beta_4$ is sufficient to promote transcription from the Fos SRE. Interestingly, while treatment with mitogens caused a significant elevation of Fos SRE activity in cells plated on the $\alpha_6\beta_4$ ligand laminin 5, it was ineffective in cells adhering to the $\alpha_2\beta_1$ ligand collagen I, indicating that the expression of Fos in response to mitogens requires ligation of a specific integrin, such as $\alpha_6\beta_4$. Future studies will be required to examine further the mechanism by which $\alpha_6\beta_4$ controls immediate-early

gene expression. For example, it is known that Rho family proteins can activate the Fos promoter by stimulating the serum response factor (Hill *et al.*, 1995). The ability of $\alpha_6\beta_4$ to stimulate Rac may thus contribute to the activation of Fos promoter in response to laminin 5. In addition, since it is well established that Jnk controls the activity of the Jun promoter (Karin, 1995), it is likely that $\alpha_6\beta_4$ also regulates the expression of the immediate-early expression gene Jun. Taken together, these observations suggest that $\alpha_6\beta_4$ is a crucial regulator of immediate-early gene expression.

What is the biological significance of $\alpha_6\beta_4$ signaling? The results of our cell proliferation analysis indicate that $\alpha_6\beta_4$ signaling promotes transit through G_1 in keratinocytes and other $\alpha_6\beta_4$-expressing cells exposed to mitogens. In this respect, $\alpha_6\beta_4$ appears to be functionally distinct from other integrins, such as $\alpha_3\beta_1$ and $\alpha_2\beta_1$, which do not appear to be able to do so. In fact, it is quite remarkable that keratinocytes plated on the $\alpha_2\beta_1$ ligand collagen I adhere and spread but do not enter into the S phase despite being exposed to otherwise mitogenic concentrations of EGF. We have observed recently that a class of β_1 and α_v integrins, which include $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$, are also linked to the Ras–Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996). Taken together, these observations suggest that the ability of extracellular matrix to control cell proliferation, thereby mediating anchorage-dependent cell growth, depends on its composition and the repertoire of integrins on the responding cell.

What is the mechanism by which $\alpha_6\beta_4$ signaling regulates cell proliferation? Previous studies have indicated that the cell cycle of normal cells contains an anchorage-dependent transition in early–mid G_1. In fact, adhesion to the extracellular matrix is required for translation of cyclin D_1 in cells exposed to mitogens, suggesting that integrin- and growth factor-dependent signals converge prior to the induction of cyclin D_1 to control progression through G_1 (Fang *et al.*, 1996; Zhu *et al.*, 1996). Our results suggest that these signals are integrated before the induction of immediate-early gene expression. The simplest hypothesis is that in normal cells growth factor receptors and specific integrins cooperate to activate MAP kinase beyond the threshold level required for immediate-early gene expression. Since most dominant oncogenes, including Shc (Pelicci *et al.*, 1992), induce neoplastic transformation by constitutively activating the Ras–MAP kinase pathway, this model also explains why neoplastic cells usually display anchorage-independent growth.

In conclusion, the results of this study indicate that the coupling of $\alpha_6\beta_4$ integrin to Ras–Erk and Rac–Jnk pathways mediated by Shc regulates immediate-early gene expression and cell cycle progression in response to mitogens. Since the major keratinocyte integrins, in addition to $\alpha_6\beta_4$, are $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which are not coupled to Shc, the signaling function of $\alpha_6\beta_4$ may explain why cell proliferation is restricted to the basal layer in the epidermis and other stratified epithelia. In addition, since exit from the cell cycle is a prerequisite for differentiation, our results may also explain why the onset of keratinocyte differentiation is coupled to the detachment from the basement membrane (Hall and Watt, 1989). Finally, the ability of $\alpha_6\beta_4$ to stimulate cell growth suggests that its overexpression in squamous carcinoma (Kimmel and Carey, 1986; Wolf *et al.*, 1990) may contribute to tumor progression.

Materials and methods

Antibodies and extracellular matrix molecules

The specificity of anti-β_4 Mab 3E1 was described previously (Giancotti *et al.*, 1992). The polyclonal antiserum to the β_4 ectodomain was generated by immunizing a rabbit with a GST fusion protein comprising amino acids 31–217. The inhibitory anti-β_4 Mab A9 was obtained from Tom Carey (Comprehensive Cancer Center, University of Michigan at Ann Arbor). Hybridomas producing the anti-α_1 Mab TS2/7 and anti-β_1 Mab TS2/16 were obtained from ATCC (Rockville, MD). The anti-α_2 Mab P1E6, anti-α_3 Mab P1B5 and anti-α_5 Mab P1D6 were from Gibco-BRL (Gaithersburg, MD). The anti-β_1 Mab 4B4 was from Coulter (Hialeah, FL). The anti-MHC Mab W6.32 reacts with human and cultured rodent cells. The anti-FLAG M2 Mab and anti-HA peptide tag Mab 12CA5 were purchased from Eastman Kodak Company (New Haven, CT) and Boehringer Mannheim (Indianapolis, IN), respectively. The anti-Shc Mab and the recombinant horseradish peroxidase (HRP)-conjugated anti-P-Tyr Mab RC20 were from Transduction Laboratories (Lexington, KY). Anti-Erk2 and anti-Grb2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human fibronectin and collagen I were purchased from Gibco-BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg *et al.*, 1993; Spinardi *et al.*, 1995).

Cell lines, constructs and transfections

HeLa cells were cultured in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (FCS). Primary human keratinocytes were cultured in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 μg/ml) and human recombinant EGF (5 ng/ml) (Gibco BRL). NIH 3T3 cells expressing recombinant human β_4 were maintained in DMEM containing 10% bovine calf serum (BCS) and 3 mM L-histidinol (Sigma, St Louis, MO). Primary dermal fibroblasts were obtained from Clonetics (San Diego, CA) and cultured in fibroblast basal medium (FBM) supplemented with 2% FCS, 2 ng/ml bFGF and 10 μg/ml insulin.

To generate pLXSHD-β_4, the full-length human β_4 cDNA was subcloned in the *Eco*RI site of pLXSHD, a derivative of the Moloney leukemia virus-derived retroviral vector pLXSN containing as a selection marker the histidinol dehydrogenase gene (Dusty Miller and Rosman, 1989). The recombinant virus was produced by transiently transfecting 293-T cells with 10 μg of pLXHD-β_4 and 10 μg of packaging-defective ecotropic virus (Landau and Littman, 1992). Subconfluent NIH 3T3 cells were infected with a dilution of the culture supernatant collected 48 h after transfection. Cell lines expressing recombinant human β_4 were isolated by L-histidinol selection and identified by FACS analysis. Rat 804G cells expressing a recombinant wild-type human β_4 subunit were previously described (Spinardi *et al.*, 1993). To generate a cytomegalovirus (CMV)-driven eukaryotic expression vector encoding a tail-less β_4 subunit, the 2.3 kb *Eco*RI–*Sca*I fragment of β_4 cDNA was ligated into *Eco*RI–*Bam*HI-digested pRK-5. The 2.3 kb *Eco*RI–*Xba*I fragment of the resulting plasmid was then ligated into *Eco*RI–*Xba*I-linearized pRc-CMV. The recombinant β_4 subunit encoded by this vector is truncated immediately after Lys734, which marks the boundary between the transmembrane and intracellular domains of the polypeptide. Clone L cells were generated by transfecting parental 804G cells with the above vector according to previously published protocols (Spinardi *et al.*, 1993). FACS analysis was used to verify that clone A and clone L cells had comparable levels of expression of recombinant β_4. Metabolic labeling with [^{35}S]methionine/cysteine (Translabel, ICN, Costa Mesa, CA) and immunoprecipitation were used to verify the correct assembly of recombinant β_4 with endogenous α_6 in both NIH 3T3 and 804G transfectants.

The CMV promoter-based expression vectors encoding HA-tagged Erk2, dominant-negative p52^{Shc} (Y317F) and dominant-negative Ras (N17) were obtained from Edward Scolnik (NYU School of Medicine). The vectors encoding GST–Jun and Flag-tagged Jnk1 were described previously (Hibi *et al.*, 1993; Derijard *et al.*, 1994). The cDNAs encoding dominant-negative RhoA (N19) and Rac1 (N17) (Khosravi-Far *et al.*, 1995) were subcloned in pcDNA3. Dominant-negative Cdc42 (N17) in pCMV5 was obtained from Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). The Fos-SRE–Luc reporter plasmid was from

Joseph Schlessinger. Vectors were transiently transfected in HeLa and NIH 3T3 cells by the lipofectamine method (Gibco-BRL).

Biochemical methods

To obtain ligation of integrins in the absence of any co-stimulus, the cells were growth factor starved for 36 h, detached, and resuspended in serum-free medium. The cells were then either incubated in suspension with polystyrene beads (2.5 μm diameter, IDC, Portland, OR) coated with anti-integrin Mabs (Mainiero *et al.*, 1995; Wary *et al.*, 1996) or plated onto dishes coated sequentially with affinity-purified goat anti-mouse IgGs and anti-integrin Mabs or extracellular matrix proteins. At the coating concentrations used, the cells attached and spread equally well on laminin 5 and collagen I, attached and partially spread on the anti-β_4 Mab 3E1, and attached without spreading on poly-L-lysine and the control Mab W6.32. As a positive control for Jnk activation, adherent cells were exposed to UV radiation as previously described (Hibi *et al.*, 1993; Derijard *et al.*, 1994). At the end of the incubation, the cells were extracted and subjected to biochemical analysis.

To immunoprecipitate Shc and $\alpha_6\beta_4$, primary human keratinocytes were extracted in Triton lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.01% aprotinin, 4 mg/ml pepstatin A, 10 mg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (PMSF) (all from Sigma) for 30 min on ice. Immunoprecipitation, SDS–PAGE and immunoblotting analysis were performed as previously described (Giancotti and Ruoslahti, 1990; Mainiero *et al.*, 1995). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

To examine Erk activity, cells were extracted with NP-40 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing phosphatase and protease inhibitors for 30 min on ice. Endogenous and recombinant tagged Erks were immunoprecipitated with anti-Erk2 or anti-HA Mab, respectively, and subjected to *in vitro* kinase assay. The kinase reaction was initiated by adding to the beads 25 μl of kinase buffer (25 mM Tris pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 20 μM cold ATP, 0.5 mM sodium orthovanadate) containing 5 μCi of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Biomedicals Inc.) and 2.5 μg of myelin basic protein (Sigma). After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS–PAGE.

To analyze the activation of Jnk, cells were extracted for 30 min on ice with modified Triton lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol) containing phosphatase and protease inhibitors. Endogenous Jnk was precipitated with 3 μg of GST–Jun fusion protein coupled to glutathione–agarose beads. After washing, the beads were incubated with 25 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP. Recombinant Flag-tagged Jnk1 was immunoprecipitated with the anti-Flag Mab M2 and incubated with 25 μl of kinase buffer containing 2.5 μg of GST–Jun and 5 μCi of [γ-^{32}P]ATP. After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS–PAGE.

To estimate Ras activation, primary human keratinocytes were starved for 48 h in K-SFM and labeled for 12 h with [^{32}P]orthophosphate (0.5 mCi/ml, ICN) in phosphate-free DMEM supplemented with 0.1% phosphate-free BCS. After stimulation, the cells were extracted and the samples subjected to Ras-GTP loading assay as described previously (Gale *et al.*, 1993). Nucleotides bound to Ras were analyzed by TLC on PEI-cellulose plates in 0.75 M K_2HPO_4, pH 3.5. Radioactivity in GDP and GTP was estimated by Phosphorimager analysis.

To measure trascription from the Fos SRE, HeLa cells and NIH 3T3 transfectants were transiently transfected with the reporter plasmid Fos-SRE–Luc. After 24 h of growth factor starvation, the cells were detached and plated on dishes coated with laminin 5, collagen I or poly-L-lysine for 30 min. The HeLa cells were then either left untreated or exposed to 50 ng/ml EGF, 20 ng/ml PDGF-BB, 2 ng/ml bFGF and 10 μg/ml insulin for 10 min. The NIH 3T3 transfectants were either left untreated or exposed to 10 ng/ml PDGF-BB, 5 ng/ml bFGF and 10 μg/ml insulin for 10 min. Luciferase activity in cell lysates was estimated as previously described (Brasier *et al.*, 1989).

Measurement of cell cycle progression

To monitor progression through G_1 and entry into S phase, the cells were starved by incubation in medium devoid of serum and growth factors for 48 h, detached and plated at low density on microtiter plates or glass coverslips coated with 10 μg/ml poly-L-lysine, laminin 5, collagen I or a mixture of 10 μg/ml fibronectin and 10 μg/ml poly-L-lysine. The keratinocytes were incubated in K-SFM supplemented with 5 ng/ml human recombinant EGF. The NIH 3T3 transfectants were incubated in FBM supplemented with 20 ng/ml PDGF, 2 ng/ml bFGF and 10 μg/ml insulin. Primary dermal fibroblasts were incubated in FBM with 2 ng/ml bFGF and 10 μg/ml insulin. The media were supplemented with 10 μM BrdU and, when indicated, with the inhibitory anti-β_1 Mab 4B4, the inhibitory anti-β_4 Mab A9 or the control anti-MHC Mab W6.32. After 22 h of incubation, the cells were fixed in 70% ethanol, 50 mM glycine, pH 2.0 for 30 min at –20°C and stained with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN). The percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields.

Acknowledgements

We thank Dafna Bar-Sagi, Tom Carey, Jonathan Chernoff, Maria Galisteo, Ed Scolnik and Jossie Schlessinger for constructs and antibodies, Mitchell Yeon for expert technical assistance, and Giuseppe Pintucci for Phosphorimager analysis. We are especially grateful to Michael Dans for generating clone L cells. This work was supported by NIH grant CA 58976 and DAMD grant 17-94-J4306. F.M. was supported by a fellowship from the American Italian Foundation for Cancer Research. C.M. is on leave of absence from the Istituto Nazionale della Nutrizione (Rome, Italy). F.G.G. was the recipient of awards from the Lucille P.Markey and the Irma T.Hirschl Charitable Trusts and is an Established Investigator of the American Heart Association.

References

Adams,J.C. and Watt,F.M. (1993) Regulation of development and differentiation by extracellular matrix. *Development*, **117**, 1183–1198.

Bar-Sagi,D., Rotin,D., Batzer,A., Mandiyan,V. and Schlessinger,J. (1993) SH3 domains direct localization of signaling molecules. *Cell*, **74**, 83–91.

Brasier,A.R., Tate,J.E. and Habener,J.F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques*, **7**, 1116–1122.

Carter,W.G., Kaur,P., Gil,S.G., Gahr,P.J. and Wayner,E.A. (1990a) Distinct functions for integrins $\alpha_3\beta_1$ in focal adhesions and $\alpha_6\beta_4$/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.*, **111**, 3141–3154.

Carter,W.G., Wayner,E.A., Bouchard,T.S. and Kaur,P. (1990b) The role of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in cell–cell and cell–substrate adhesion of human epidermal cells. *J. Cell Biol.*, **110**, 1387–1404.

Carter,W.G., Ryan,M.C. and Gahr,P.J. (1991) Epiligrin, a new cell adhesion ligand for integrin $\alpha_3\beta_1$ in epithelial basement membranes. *Cell*, **65**, 599–610.

Clark,E.A. and Brugge,J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233–239.

Derijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1038.

Dowling,J., Yu,Q.-C. and Fuchs,E. (1996) β_4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.*, **134**, 559–572.

Dusty Miller,A. and Rosman,G.J. (1989) Improved retroviral vectors for gene transfer expression. *Biotechniques*, **9**, 980–988.

Einheber,S., Milner,T.A., Giancotti,F.G. and Salzer,J.L. (1993) Axonal regulation of Schwann cell integrin expression suggests a role for $\alpha_6\beta_4$ in myelination. *J. Cell Biol.*, **123**, 1223–1236.

Fang,F., Orend,G., Watanabe,N., Hunter,T. and Ruoslahti,E. (1996) Dependence of cyclin E–CDK 2 kinase activity on cell anchorage. *Science*, **271**, 499–502.

Gale,N.W., Kaplan,S., Lowenstein,E.J., Schlessinger,J. and Bar-Sagi,D. (1993) Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature*, **363**, 88–92.

Giancotti,F.G. (1996) Signal transduction by the $\alpha_6\beta_4$ integrin: charting the path between laminin binding and nuclear events. *J. Cell Sci.*, **109**, 1165–1172.

Giancotti,F.G. and Mainiero,F. (1994) Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta*, **1198**, 47–64.

Giancotti,F.G. and Ruoslahti,E. (1990) Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell*, **60**, 849–859.

Giancotti,F.G., Stepp,M.A., Suzuki,S., Engvall,E. and Ruoslahti,E. (1992) Proteolytic processing of endogenous and recombinant beta 4 integrin subunit. *J. Cell Biol.*, **118**, 951–959.

Green,H. (1977) Terminal differentiation of cultured human epidermal cells. *Cell*, **11**, 405–416.

Hall,P.A. and Watt,F.M. (1989) Stem cells: the generation and maintenance of cellular diversity. *Development*, **106**, 619–633.

Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) Identification of an oncoprotein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135–2148.

Hill,C.S., Wynne,J. and Treisman,R. (1995) The Rho family GTPase RhoA, Rac1 and Cdc42Hs regulate transcriptional activation by SRF. *Cell*, **81**, 1159–1170.

Hogervorst,F., Kuikman,I., von dem Borne,A.E.G.Kr. and Sonnenberg,A. (1990) Cloning and sequence analysis of β_4 cDNA: an integrin subunit that contains a unique 118 kDa cytoplasmic domain. *EMBO J.*, **9**, 745–770.

Joneson,T., Withe,M.A., Wigler,M.H. and Bar-Sagi,D. (1996) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of Ras. *Science*, **271**, 810–812.

Kajiji,S., Tamura,R.N. and Quaranta,V. (1989) A novel integrin ($\alpha_E\beta_4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.*, **8**, 673–680.

Karin,M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.*, **270**, 16483–16486.

Kennel,S.J., Godfrey,V., Chang,L.Y., Lankford,T.K., Foote,L.J. and Makkinje,A. (1992) The β_4 subunit of the integrin family is displayed on a restricted subset of endothelium in mice. *J. Cell Biol.*, **101**, 145–150.

Khosravi-Far,R., Solski,P.A., Kinch,M.S., Burridge,K. and Der,C.J. (1995) Activation of Rac1, RhoA and mitogen activated protein kinases is required for Ras transformation. *Mol. Cell Biol.*, **15**, 6443–6453.

Kimmel,K.A. and Carey,T.E. (1986) Altered expression in squamous carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.*, **46**, 3614–2623.

Klein,S., Giancotti,F.G., Presta,M., Albelda,S.M., Buck,C.A. and Rifkin,D.B. (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell*, **4**, 973–982.

Klippel,A., Reinhard,C., Kavanaugh,W.M., Apell,G., Escobedo,M.-A. and Williams,L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.*, **16**, 4117–4127.

Landau,N.R. and Litmann,D.R. (1992) Packaging system for rapid production of murine leukemia virus vectors with variable tropism. *J. Virol.*, **66**, 5110–5113.

Lange-Carter,C.A. and Johnson,G.L. (1994) Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science*, **265**, 1458–1461.

Lemmon,M.A., Ferguson,K.M. and Schlessinger,J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, **85**, 621–624.

Lin,C.Q. and Bissell,M.J. (1993) Multi-faceted regulation of cell differentiation via extracellular matrix. *FASEB J.*, **7**, 737–743.

Mainiero,F., Pepe,A., Wary,K.K., Spinardi,L., Mohammadi,M., Schlessinger,J. and Giancotti,F.G. (1995) Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO J.*, **14**, 4470–4481.

McNamee,H.P., Ingber,D.E. and Schwartz,M.A. (1993) Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.*, **121**, 673–678.

Minden,A., Lin,A., Claret,F.-X., Abo,A. and Karin,M. (1995) Selective activation of the Jnk signaling cascade and c-Jun transcriptional activity by the small GTPase Rac and Cdc42Hs. *Cell*, **81**, 1147–1157.

Niessen,C.M., Hogervorst,F., Jaspars,L.H., De Melker,A.A., Delwel,G.O., Hulsman,E.H.M., Kuikman,I. and Sonnenberg,A. (1994) The $\alpha_6\beta_4$ integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.*, **2111**, 360–367.

Nobes,C.D., Hawkins,P., Stephens,L. and Hall,A. (1995) Activation of the small GTP-binding proteins rho and rac by growth factor receptor. *J. Cell Sci.*, **108**, 225–233.

Pawson,T. (1995) Protein modules and signaling networks. *Nature*, **373**, 573–580.

Pelicci,G., Lanfrancone,L., Grignani,F., McGlade,J., Cavallo,F., Forni,G., Nicoletti,I., Grignani,F., Pawson,T. and Pelicci,P.G. (1992) A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, **70**, 93–104.

Qiu,R.-G., Chen,J., Kirn,D., McCormick,F. and Symons,M. (1995) An essential role for Rac in Ras transformation. *Nature*, **374**, 457–459.

Rodriguez-Viciana,P., Warne,P.H., Dhand,R., Vanhaesebroek,B., Gout,I., Fry,M.J., Waterfield,M.D. and Downard,J. (1994) Phosphatidylinositol-3-OH kinase as direct target of ras. *Nature*, **370**, 527–532.

Rousselle,P., Lunstrum,G.P., Keene,D.R. and Burgeson,R.E. (1991) Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.*, **114**, 567–576.

Schlessinger,J. (1994) SH2/SH3 signaling proteins. *Curr. Opin. Genet. Dev.*, **4**, 25–30.

Schwartz,M.A., Shaller,M.D. and Ginsberg,M.H. (1995) Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.*, **11**, 549–599.

Sonnenberg,A., Linders,C.J.T., Daams,J.H. and Kennel,S.J. (1990) The $\alpha_6\beta_1$(VLA-6) and $\alpha_6\beta_4$ protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.*, **96**, 207–217.

Sonnenberg,A., de Melker,A.A., Martine de Velasco,A.M., Janssen,H., Calafat,J. and Niessen,C.M. (1993) Formation of hemidesmosomes in cells of a transformed murine cell line and mechanisms involved in adherence of these cells to laminin and kalinin. *J. Cell Sci.*, **106**, 1083–1102.

Spinardi,L., Ren,Y.-L., Sanders,R. and Giancotti,F.G. (1993) The β_4 subunit cytoplasmic domain mediates the interaction of $\alpha_6\beta_4$ integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell*, **4**, 871–884.

Spinardi,L., Einheber,S., Cullen.,T., Milner,T.A. and Giancotti,F.G. (1995) A recombinant tail-less integrin $\alpha_6\beta_4$ subunit disrupts hemidesmosomes, but does not suppress $\alpha_6\beta_4$-mediated cell adhesion to laminins. *J. Cell Biol.*, **129**, 473–487.

Stepp,M.A., Spurr-Michaud,S., Tisdale,A., Elwell,J. and Gipson,I.K. (1990) Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl Acad. Sci. USA*, **87**, 8970–8974.

Suzuki,S. and Naitoh,Y. (1990) Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.*, **9**, 757–763.

Treisman,R. (1995) Journey to the surface of the cell: Fos regulation and SRE. *EMBO J.*, **14**, 4905–4913.

Wadsworth,S., Halvorson,M.J. and Coligan,J.E. (1992) Developmentally regulated expression of the β_4 integrin on immature mouse thymocytes. *J. Immunol.*, **149**, 421–428.

Wary,K.K., Mainiero,F., Isakoff,S.J., Marcantonio,E.E. and Giancotti,F.G. (1996) The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell*, **87**, 733–743.

Wolf,G.T., Carey,T.E., Schmaltz,S.P., McClatchey,K.D., Poore,J., Glaser,L., Hayashida,D.J.S. and Hsu,S. (1990) Altered antigen expression predicts outcome in squamous cell carcinomas of the head and neck. *J. Natl Cancer Inst.*, **82**, 1566–1572.

Wymann,M.P., Bulgarelli-Leva,G., Zvelebil,M.J., Pirola,L., Vanhaesebroeck,B., Waterfield,M.D. and Panayotou,G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.*, **16**, 1722–1733.

Xia,Y., Gil,S.G. and Carter,W.G. (1996) Anchorage mediated by integrin $\alpha_6\beta_4$ to laminin 5 (epiligrin) regulates tyrosine phosphorylation of a membrane-associated 80-kD protein. *J. Cell Biol.*, **132**, 727–740.

Zhu,X., Ohtsubo,M., Bohmer,R., Roberts,J.M. and Assoian,R.K. (1996) Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E–cdk2 and phosphorylation of the retinoblastoma protein. *J. Cell Biol.*, **133**, 391–403.

Received on August 26, 1996; revised on January 9, 1997

Integrin signaling: specificity and control of cell survival and cell cycle progression

Filippo G Giancotti

Integrin-mediated adhesion to the extracellular matrix plays an important role in regulating cell survival and proliferation. There is now increasing evidence that integrins activate shared as well as subgroup-specific signaling pathways. The signals from these adhesion receptors are integrated with those originating from growth factor and cytokine receptors in order to organize the cytoskeleton, stimulate mitogen-activated protein kinase cascades, and regulate immediate early gene expression. The repertoire of integrins and composition of the extracellular matrix appear to dictate whether a cell will survive, proliferate or exit the cell cycle and differentiate in response to soluble factors.

Addresses
Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center and the Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, Box 216, 1275 York Avenue, New York, NY 10021, USA; e-mail: f-giancotti@ski.mskcc.org

Current Opinion in Cell Biology 1997, 9:691–700

http://biomednet.com/elecref/0955067400900691

Abbreviations

ECM	extracellular matrix
ERK	extracellular signal regulated kinase
FAK	focal adhesion associated kinase
ILK	integrin-linked kinase
JNK	Jun amino-terminal kinase
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
PI-3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
SH	Src homology
TAM	tyrosine activation motif

Introduction

It has been known for a long time that normal cells need to adhere to serum-derived extracellular matrix (ECM) components, such as fibronectin and vitronectin, in order to proliferate *in vitro*. The loss of this requirement is a hallmark of neoplastic cells and represents perhaps the best correlate to *in vivo* tumorigenesis. These findings have led to the hypothesis that the ECM generates signals necessary for the progression of normal cells through the cell cycle and the speculation that these signals may be constitutively active in neoplastic cells (reviewed in [1]). Subsequent studies have revealed that, upon interacting with specific ECM components, some cell types exit from the cell cycle and undergo morphological and functional differentiation even in presence of otherwise mitogenic concentrations of growth factors. For example, while myoblasts proliferate and remain undifferentiated on fibronectin, on laminin they cease growing and fuse to form myotubes. Endothelial cells display similar behavior: they proliferate on fibronectin, but are induced to form capillary-like structures when confronted with a laminin-rich basement membrane gel. Finally, fibronectin, which promotes proliferation of the above-mentioned cell types, promotes differentiation in erythroblasts (reviewed in [2]). These observations suggest that the ECM can have divergent effects on cellular behavior depending on its composition and the cell type involved.

Since their original discovery about a decade ago, it has become clear that the integrins not only mediate cell adhesion to the ECM, but also activate intracellular signaling pathways. Each integrin consists of an α and a β subunit, and in mammals we know of 16 distinct α subunits and 8 β subunits which variously combine to form 22 receptors, each characterized by a distinct, although largely overlapping, ligand-binding specificity (reviewed in [3,4]). Despite this high degree of apparent redundancy, most integrins seem to have specific biological functions during development (reviewed in [5]), raising the possibility of signaling differences between integrins.

In this review, I will focus on the membrane-proximal events and major signaling pathways activated by the ECM in mammalian fibroblasts and epithelial cells, and discuss the mechanisms by which these signaling events regulate cytoskeletal organization, progression through the G_1 phase of the cell cycle, cell survival, and cell differentiation.

Focal adhesion kinase

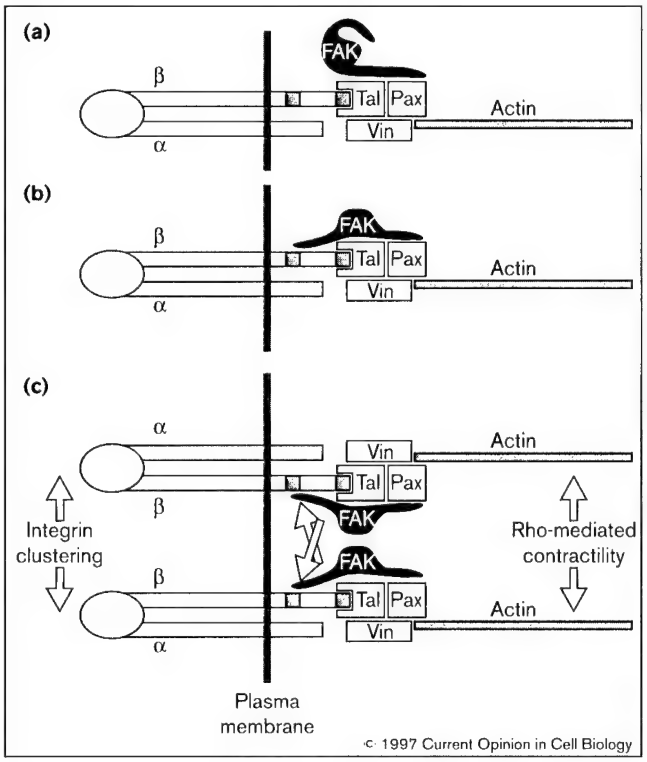
All β1 and αv subunit containing integrins share the ability to promote the assembly of focal adhesions and, at the same time, activate the focal adhesion associated kinase, FAK. FAK is an unusual nonreceptor tyrosine kinase which consists of a central catalytic domain flanked by amino-terminal and carboxy-terminal domains devoid of the Src homology (SH)2 or SH3 domains that are characteristic of other cytoplasmic tyrosine kinases. In contrast to its closest relative, Pyk2, which displays a restricted tissue distribution, FAK is widely expressed and also appears to be activated by those growth factor and cytokine receptors that affect the cytoskeleton (reviewed in [6]).

Activation

Although the mechanism by which integrins activate FAK is incompletely understood, it is clear that this process is tightly coupled to the process of assembly of focal adhesions and associated stress fibers. The

activation of FAK requires the same segment of the β integrin subunit cytoplasmic domain that is thought to interact with talin and mediate the incorporation of integrins in focal adhesions [7]. Talin binds directly to the carboxy-terminal domain of FAK [8], and also interacts with vinculin and thereby paxillin [9]. Paxillin in turn binds to a distinct site in the carboxyl terminus of FAK [10]. Thus, it is possible that the initial recruitment of FAK to activated integrins is indirect and mediated by talin. In apparent contrast with this hypothesis, it has been reported that the amino-terminal segment of FAK interacts directly *in vitro* with a portion of the integrin β subunit cytoplasmic domain that is distinct from the talin-binding site [11]. Figure 1 illustrates a model that reconciles these seemingly contradictory observations. Upon being recruited to nascent focal adhesions by talin, FAK would undergo a conformational change and interact through its amino-terminal domain with the integrin β subunit tail. As the amino terminus of FAK plays a negative autoregulatory role, possibly by folding back onto the catalytic domain [12], this conformational transition may be a prerequisite for FAK's catalytic activity. The aggregation of integrins that is a consequence of their binding to extracellular ligand would then result in a correspondent oligomerization of FAK. The kinase would finally be activated by a *trans*-autophosphorylation mechanism similar to that established for receptor tyrosine kinases. Although it needs validation, this model is appealing because it explains why the activation of FAK by stimuli such as lysophosphatidic acid (LPA), which acts on Rho and thereby enhances actin filament contractility, requires integrin ligation [13].

Figure 1



Model of FAK recruitment and activation. **(a)** The binding of ligand (shaded oval) to the integrin (α and β) ectodomain promotes, presumably via a conformational change, the interaction of the β integrin subunit tail with talin (Tal) and, via talin, with vinculin (Vin) and paxillin (Pax). Vinculin and paxillin interact with the actin cytoskeleton. FAK is recruited to the nascent complex via the interaction of its carboxy-terminal domain with paxillin and talin. **(b)** FAK undergoes a conformational change which allows it to interact through its amino-terminal domain with a membrane-proximal segment of the β integrin tail. **(c)** The geometry of integrin-binding sites in the ECM and Rho-mediated contractility cooperate to promote integrin clustering, thereby bringing different FAK molecules together. FAK then undergoes *trans*-autophosphorylation (indicated by crossed arrows). The shaded squares in the integrin β subunit tail correspond to the FAK- and talin-binding sites.

Target effectors

The activation of FAK has been linked to a number of molecular events. Activated FAK undergoes autophosphorylation at tyrosine residue 397 and thereby binds to the SH2 domain of Src or Fyn [14]. The Src family kinase (Src or Fyn) then phosphorylates a number of FAK-associated proteins, including paxillin, tensin and $p130^{CAS}$ [15•,16]. While the phosphorylation of paxillin and tensin may be involved in the regulation of focal adhesions, $p130^{CAS}$ is a multidomain docking protein and, upon phosphorylation, interacts with the adaptor proteins Crk and Nck, thereby potentially regulating signaling to the mitogen-activated protein kinases (MAPKs) extracellular signal regulated kinase (ERK) and Jun amino-terminal kinase (JNK) [16,17]. Src can also phosphorylate FAK at tyrosine residue 925, creating a binding site for the Grb2–mSOS complex, but the stoichiometry of this event appears to be low [18]. Finally, autophosphorylated FAK can combine with and activate phosphoinositide 3-kinase (PI-3K) [19]. FAK is thus linked to a number of intracellular signaling pathways that are potentially able to modify the cytoskeleton and influence transcriptional activity in the nucleus.

Role in cell migration and cell survival

What is the biological function of FAK? Although there is evidence suggesting that FAK can, upon overexpression or association with elevated levels of Src, activate the Ras–ERK signaling pathway in response to cell adhesion [12,17], it is unclear if FAK physiologically plays this role. In fact, recent studies have provided evidence that, in normal fibroblasts and endothelial cells, the activation of ERK in response to integrin ligation is mediated by the adaptor protein Shc independently of FAK [20••]. Accordingly, the introduction of a dominant-negative version of FAK does not impair the activation of ERK caused by cell adhesion to fibronectin in these cells [21•]. Although these findings do not support the hypothesis that FAK can signal through ERK in normal cells, it remains possible that FAK plays such a role upon overexpression in carcinomas [22].

The analysis of FAK knockout mice has provided important information on the biological function of FAK.

Embryonic fibroblasts derived from these mice form numerous centrally located small focal contacts, but fail to form the large peripheral focal adhesions that may be required at the leading edge of the cell to propel migration; accordingly, these cells migrate less efficiently than control cells [23]. The observations that overexpression of FAK increases cell migration [24] while a dominant-negative form of the kinase inhibits it [25] are also consistent with a role of FAK in the dynamic regulation of focal adhesions during cell migration. Finally, recent data have indicated that a constitutively active form of FAK can promote anchorage-independent survival and growth in epithelial cells [26•], suggesting that FAK may also contribute to cell survival and proliferation.

Other membrane-proximal kinases

Integrin signaling is likely to involve other kinases in addition to FAK and Src family kinases. A recent report has provided evidence that cell adhesion to various ECM components activates Abl, a tyrosine kinase with different functions in the cytoplasm and in the nucleus [27]. Cytoplasmic Abl is likely to be incorporated in focal adhesions through its interaction with Mena, a homolog of the *Drosophila* Abl substrate Ena [28••]. As Mena contains a central proline-rich segment, which binds to profilin and may be involved in the assembly of actin filaments, Abl-mediated phosphorylation of Mena may contribute to the regulation of focal adhesions. It has also been shown that cell adhesion to the ECM is necessary for the transport of Abl from the cytoplasm to the nucleus at the $G_1 \rightarrow$S-phase boundary of the cell cycle [27]. As nuclear Abl is potentially involved in regulating the transcription of genes important for S-phase entry, this may provide a mechanism for cell cycle control by the ECM.

In contrast to FAK and Abl which are activated in response to cell adhesion, the activity of the serine/threonine-specific integrin-linked kinase (ILK) appears to be inhibited in response to integrin ligation [29•]. ILK was originally isolated by virtue of its ability to interact with the integrin β1 cytoplasmic domain in the yeast two-hybrid system and was later shown to be localized to focal adhesions [29•]. Interestingly, the amino-terminal segment of ILK contains a series of ankyrin-like repeats which may mediate association with the actin cytoskeleton, while the carboxy-terminal catalytic domain includes the region that interacts with the β1 cytoplasmic tail *in vitro*. Overexpression of ILK leads to constitutive activation of ERK and anchorage-independent cell growth [30]. However, adhesion to fibronectin decreases the activity of ILK in cells that express physiological levels of the kinase [29•]. It is thus unlikely that ILK mediates the activation of ERK and stimulation of cell cycle progression observed in response to the ECM. Rather, ILK appears to be a novel type of transforming kinase negatively regulated by cell adhesion.

Transmembrane adaptors

Recent studies have indicated that integrins can associate with different classes of transmembrane adaptors. The αvβ3 and αIIbβ3 integrins are physically and functionally associated with the integrin-associated protein (IAP), which consists of an extracellular immunoglobulin domain followed by five transmembrane helices and a short cytoplasmic tail [31]. A recent study has indicated that IAP not only cooperates with β3 integrins in binding to thrombospondin, but also activates a heterotrimeric Gi protein dependent intracellular pathway that leads to the activation of the tyrosine kinase Syk and its association with FAK [32]. These findings suggest that IAP may function in concert with β3 integrins to regulate intracellular signaling in response to thrombospondin.

The α3β1 and α6β1 integrins combine with the tetraspan proteins CD9, CD63 and CD81, which are characterized by four transmembrane segments [33]. Interestingly, CD63 and CD81 have been shown to be associated with a type II phosphatidylinositol 4-kinase (PI-4K) and may therefore control the first step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in response to cell adhesion [34].

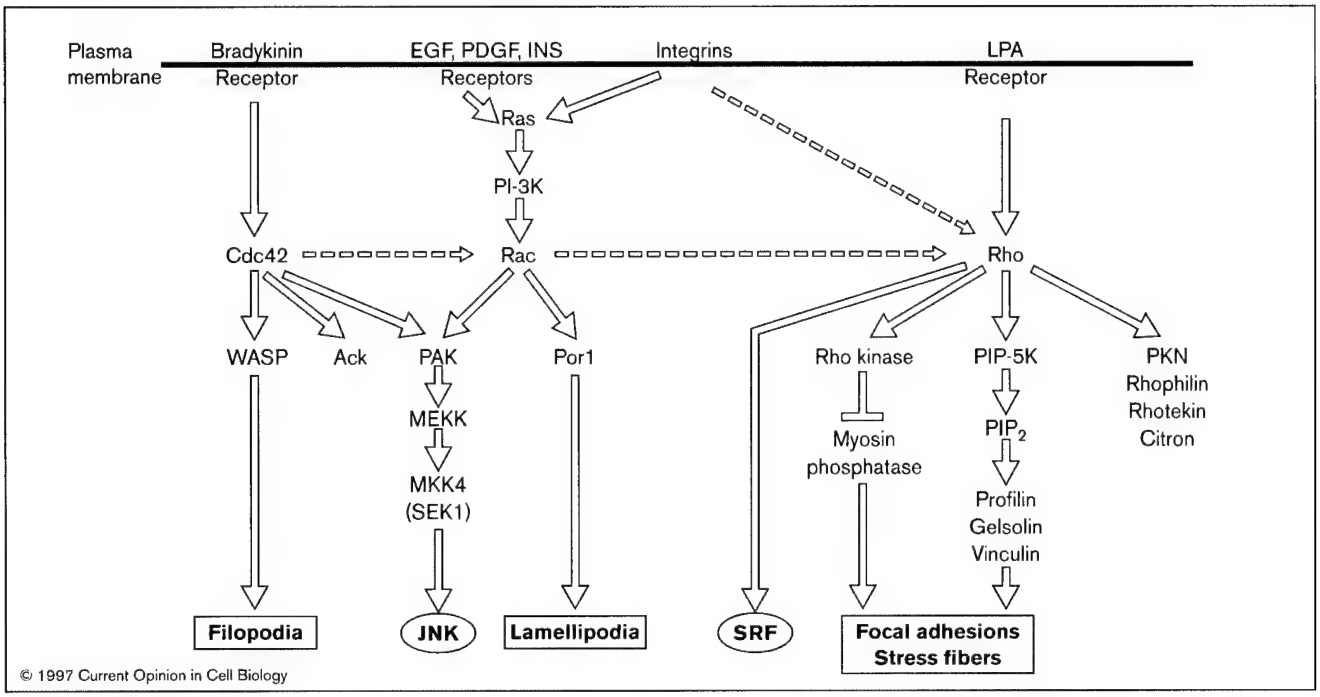
Finally, it has been observed that the two-transmembrane-domain adaptor caveolin is constitutively associated with β1 integrins in resting cells and becomes associated with the adaptor protein Shc in response to integrin ligation [20••]. Taken together, these observations suggest that various transmembrane adaptors link specific integrins to distinct intracellular signaling pathways.

Rho family GTPases

Rho family GTPases regulate the actin cytoskeleton and influence gene expression by interacting with multiple distinct target effectors (reviewed in [35]) (Figure 2). Cdc42 has been implicated in the formation of filopodia in response to bradykinin; Rac promotes the establishment of lamellipodia in response to epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin; and Rho is involved in the organization of focal adhesions and associated stress fibers in response to LPA or bombesin. In addition to these morphological effects, Rho family proteins activate MAPK cascades involved in the control of immediate early gene expression: Cdc42 and Rac are known to activate JNK, and Rho activates the serum-response factor (SRF). Finally, there is evidence that the Rho family proteins are horizontally interconnected in a hierarchical fashion: Cdc42 activates Rac, and Rac activates Rho.

In addition to being necessary for the morphological changes caused by Rho family proteins in response to growth factors [13], integrin ligation is sufficient to activate at least some of the pathways controlled by Rac and Rho. Two lines of evidence support the notion that integrins can activate Rho. First, it has been known for some time

Figure 2



Signaling via Rho family GTPases. The major signaling pathways involving Rho proteins (Cdc42, Rac and Rho), recently reviewed elsewhere [35], are illustrated diagrammatically. The integrins can activate Rac via Ras and PI-3K, and Rho through mechanisms that remain to be determined. Cdc42, Rac and Rho can also be activated by the binding of bradykinin, PDGF, EGF, INS or LPA to their receptors. Cdc42, Rac and Rho, which are 'horizontally' interconnected in a hierarchical fashion, interact with a number of target effectors. Some of these, such as WASP, Por1, Rho kinase and PIP-5K, are responsible for organizing cytoskeletal structures such as filopodia, lamellipodia and focal adhesions. Others activate elements, such as JNK and SRF, that influence immediate early gene expression. The functions of Ack, PKN, Rhophilin, Rhotekin and Citron remain to be fully established. Ack, activated Cdc42-binding kinase; EGF, epidermal growth factor; INS, insulin; MEK (not shown), MAPK/ERK kinase; MEKK, MEK kinase/SAPK kinase kinase; MKK/SEK, SAPK kinase; PAK, p21(Cdc42/Rac)-activated kinase; PDGF, platelet-derived growth factor; PIP-5K, phosphatidylinositol phosphate 5-kinase; PKN, protein kinase N; Por1, partner of Rac 1; SAPK, stress-activated protein kinase (also known as JNK); SRF, serum-response factor; WASP, Wiskott–Aldrich syndrome protein. Dotted arrows indicate pathways for which the biochemical evidence is less strong. Ovals and rectangles distinguish between molecules and structures, respectively.

that integrins can stimulate the production of PIP_2 and recent studies have provided evidence that this effect is mediated by Rho [36], possibly through its interaction with a type I isoform of phosphatidylinositol 4-phosphate 5-kinase (PIP4-5K) [37]. Second, dominant-negative Rho can partially suppress the activation of ERK in response to ligation of the α5β1 and α6β4 integrins [38,39•], suggesting that a Rho-regulated pathway may be necessary for full activation of ERK upon cell adhesion.

The observation that Rho activates a PIP4-5K [36] is intriguing. In addition to being a substrate for both PI-3K and phospholipase C-γ, and therefore being necessary for the generation of crucial lipid second messengers in response to growth factor stimulation, PIP_2 (produced from phosphatidylinositol 4-phosphate by PIP4-5K) binds to vinculin and promotes its interaction with both talin and actin [40••]. Furthermore, PIP_2 can induce the release of actin monomers from profilin and gelsolin complexes (reviewed in [41]). These events are likely to participate in the assembly of actin filaments at focal adhesions and other sites of interaction with the ECM. Rho can also activate a serine/threonine protein kinase that is homologous to myotonic dystrophy kinase, namely Rho kinase, which plays an important role in the assembly of focal adhesions [42•]. This kinase phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase, thereby suppressing the activity of the enzyme [43•]. The resulting net increase in myosin phosphorylation is expected to promote actomyosin contractility and participate in the lateral association of nascent focal complexes, an event which has been suggested to be critical for the activation of FAK [44]. These results are consistent with the previous observation that Rho is required upstream of FAK and clarify some of the mechanisms by which Rho would promote the assembly of focal adhesions and stress fibers (Figure 1).

Recent studies have provided evidence for an involvement of Rac in integrin signaling. In particular, it has been shown that ligation of the α5β1 and α6β4 integrins causes activation of JNK [39•,45]. In the case of α6β4 integrin, this event is suppressed by dominant-negative forms of Ras and Rac and by the PI3-K inhibitor wortmannin. As

PI3-K is a target effector of Ras and an activator of Rac [46•], it would appear that the activation of JNK by α6β4 integrin is mediated by the sequential activation of Ras, PI3-K and Rac. It is likely that the same and possibly additional mechanisms link α5β1 and other integrins to the activation of Rac.

The ability of integrins to control Rho family dependent pathways and thereby organize the cytoskeleton may be critical for the control of cell proliferation. Although Rho family proteins regulate immediate early gene expression, recent studies have shown that it is their ability to organize the cytoskeleton that best correlates with cell cycle progression [47••,48••]. Perhaps, as suggested by a very recent study [49], a certain degree of cytoskeletal organization is required to orient the signal transduction machinery of the cell in such a way that it can respond to biochemical signals from the ECM and soluble mitogens.

Role of α6β4 integrin signaling in assembly of hemidesmosomes

In contrast to β1 and αv integrins which participate in the formation of focal adhesions, the α6β4 integrin, which is characterized by the uniquely large cytoplasmic domain of the β4 subunit, is involved in the assembly of hemidesmosomes. The hemidesmosomes are punctuate junctions that stabilize the adhesion of stratified and complex epithelia to the basement membrane. In contrast to focal adhesions, which are linked to the actin cytoskeleton, the hemidesmosomes are connected to the keratin filament system. The nucleation of hemidesmosomes is triggered by the binding of α6β4 integrin to laminin 5 and mediated by a signaling mechanism. Ligation of α6β4 integrin activates an integrin-associated kinase and thereby induces tyrosine phosphorylation of the β4 cytoplasmic tail [50]. This event is followed by the sequential recruitment of the adaptor molecules Shc and Grb2, which link α6β4 integrin to Ras signaling. Later on, α6β4 integrin becomes incorporated in hemidesmosomes. The β tail contains towards its carboxyl terminus two pairs of type III fibronectin-like modules interrupted by a 142 amino acid long connecting segment (Figure 3). The multiple β4 tyrosine residues that are phosphorylated *in vivo* include a tyrosine activation motif (TAM) located in the connecting segment. Interestingly, phenylalanine substitutions at the β4 TAM disrupt the association of α6β4 integrin with hemidesmosomes, but do not affect recruitment of Shc and Grb2, suggesting that the β4 TAM plays a role in the assembly of hemidesmosomes, but not the recruitment of Shc and Grb2 [50] (Figure 3).

The TAM is a bidentate phosphorylation motif consisting of two closely spaced tyrosine residues followed by a leucine at position +3, and was originally identified in the cytoplasmic tails of signaling subunits of immune receptors. It is thought that, upon phosphorylation, the immune receptor TAMs bind to the two tandem SH2 domains of tyrosine kinases, such as Syk and ZAP70, which mediate subsequent downstream signaling events (reviewed in [51]). It is possible that the phosphorylation of the β4 integrin TAM, which is a transient event, regulates the assembly of hemidesmosomes by an analogous signaling mechanism [52]. In this model, the β4 TAM mediates the recruitment of an SH2–SH2-domain adaptor, kinase, or phosphatase which directly or indirectly modifies cytoskeletal element(s) of hemidesmosomes, enabling them to bind to a different region in the β4 integrin tail. This hypothesis would also explain the previous observation that the incorporation of α6β4 integrin into hemidesmosomes requires not only the β4 TAM but also the two type III fibronectin-like modules upstream of the connecting segment [53].

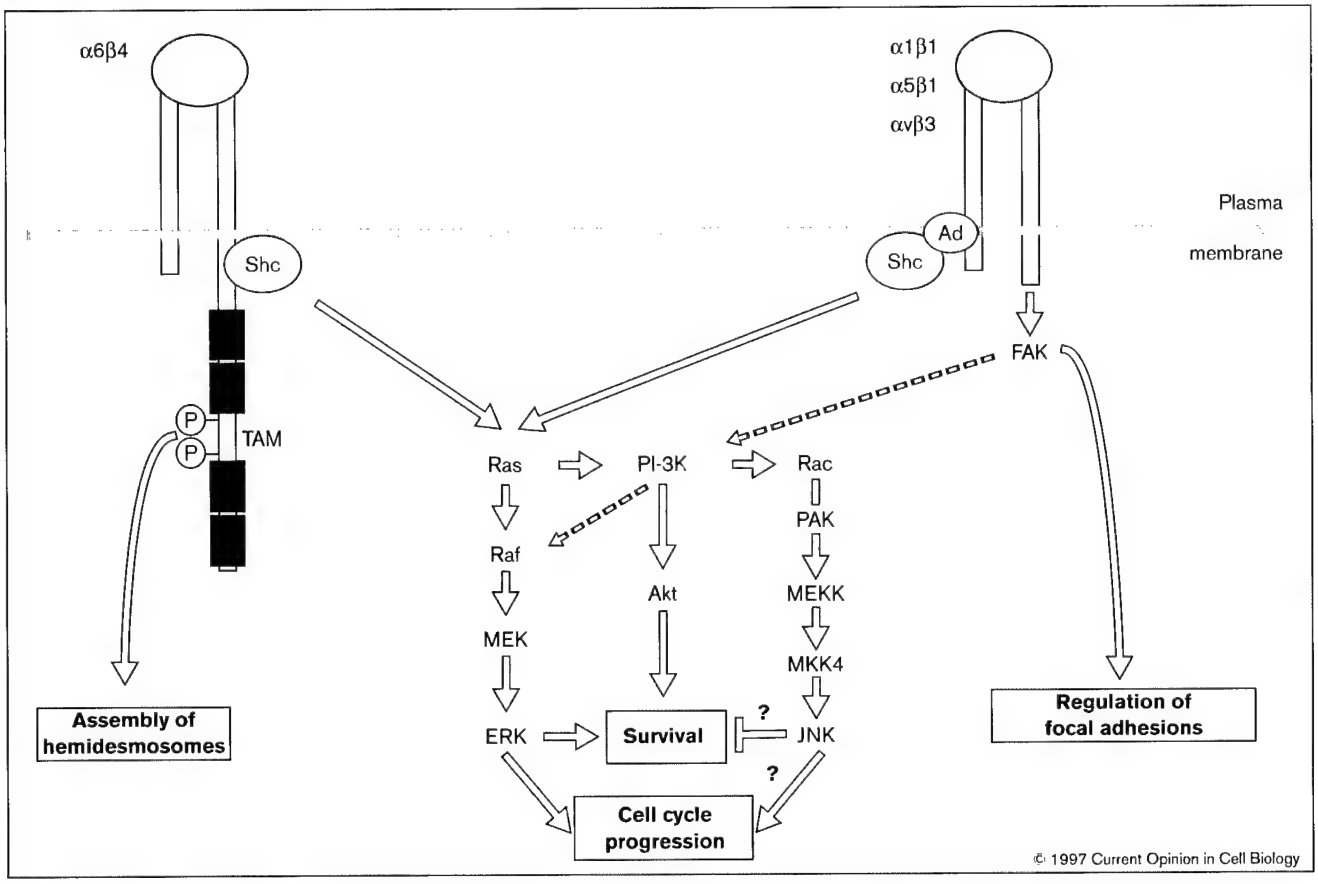
The adaptor Shc links a group of integrins to the Ras–ERK signaling cascade

Despite some initial controversy [54,55], it is now generally accepted that the activation of ERK in response to integrin ligation requires Ras signaling [12,20••]. What is the mechanism by which integrins activate Ras? Recent studies have provided evidence that certain integrins, which include the laminin receptor α6β4, the laminin/collagen receptor α1β1, the fibronectin receptor α5β1 and the broad specificity RGD (Arg–Gly–Asp)-binding receptor αvβ3, are linked to the Ras–ERK signaling pathway and the control of immediate early gene expression by the adaptor protein Shc. Other integrins, in contrast, appear to be unable to activate Ras signaling [20••,39•,50]. Shc is an SH2 and phosphotyrosine binding (PTB) domain adaptor protein which links various tyrosine-phosphorylated signal transducers to Ras. Upon recruitment to activated receptors, Shc is phosphorylated on tyrosine and binds to the Grb2–mSOS complex. This process results in the juxtaposition of the GTP exchange factor domain of mSOS to its target Ras, leading to its activation (reviewed in [56]). Although there is evidence that Shc can bind directly to the tyrosine-phosphorylated cytoplasmic domain of β4 integrin, the recruitment of Shc to activated β1 and αv integrins appears to be indirect and mediated by the interaction of the integrin α subunit with a transmembrane adaptor, possibly caveolin (Figure 3).

Control of cell cycle progression

The association of specific integrins with Shc is important for activation of the Ras–ERK signaling pathway in response to cell adhesion, while FAK plays a minor role in this process. In fact, ligation of α1β1, α5β1, and αvβ3 integrins, which are linked to Shc, results in ERK activation, but ligation of other integrins does not produce this effect, even though FAK is stimulated [20••]. In addition, cross-linking of a mutant single-chain α1 integrin subunit causes recruitment and tyrosine phosphorylation of Shc and activation of ERK without inducing FAK activation [20••]. Finally, a dominant-negative version of Shc suppresses ERK activation in response to integrin ligation [20••], but two distinct dominant-negative versions of FAK do not ([21•]; KK Wary, FG Giancotti, unpublished

Figure 3



Integrin-mediated signaling pathways. Cell survival and cell cycle progression are controlled by pathways that are largely distinct from those that are involved in the assembly of adhesive junctions such as focal adhesions or hemidesmosomes. The α6β4 integrin (left) regulates the assembly of hemidesmosomes by a mechanism which includes phosphorylation of a TAM sequence in the β4 tail and promotes progression through the G_1 phase of the cell cycle via recruitment of Shc and activation of Ras–ERK signaling. Although all β1 and αv integrins (right) can regulate focal adhesions via FAK, only some of them, including those indicated in the figure, can recruit Shc, activate Ras–ERK signaling, and promote cell cycle progression. In this case the recruitment of Shc is mediated by a transmembrane adaptor, probably caveolin. Cell survival is promoted by integrins via ERK and/or Akt. JNK may inhibit cell survival if ERK is not activated or may cooperate with ERK to promote cell cycle progression, but this hypothesis needs validation. Ad, transmembrane adaptor, possibly caveolin; MEK, MAPK/ERK kinase; MEKK, MEK kinase/SAPK kinase kinase; MKK4, SAPK kinase; PAK, p21(Cdc42/Rac)-activated kinase; SAPK, stress-activated protein kinase (also known as JNK). Circled P, phosphorylation; gray oval, integrin ligand. Dotted arrows indicate pathways for which the biochemical evidence is less strong.

data). These results indicate that Shc is necessary and sufficient to link specific integrins to the Ras–ERK signaling pathway. However, they do not exclude the possibility that FAK may cooperate with Shc to fully activate ERK in response to cell adhesion.

Two recent studies suggest a potential mechanism by which FAK could effect signaling to ERK. In the first study, it was shown that full activation of Raf, but not Ras, by mitogenic growth factors requires integrin ligation [57••]. This suggests that the activation of an integrin-dependent pathway impinging on Raf is necessary for ERK activation in response to growth factor stimulation. The second study showed that integrin ligation causes a significant activation of PI3-K, which is necessary for the activation of Raf by Ras in response to cell adhesion [58••]. Although the mechanism of PI3-K activation by integrins has not yet been fully explored, FAK may play a role in this process and thereby participate in rendering Raf responsive to Ras.

What is the biological role of the activation of Ras–ERK signaling by specific integrins? The studies on Shc signaling mentioned above have indicated that, in primary cells, engagement of integrins linked to Shc activates transcription from the Fos serum-response element (SRE) and promotes progression through the G_1 phase of the cell cycle in response to growth factors. In contrast, ligation of other integrins does not stimulate SRE-dependent transcription and results in cell cycle arrest even in the presence of otherwise mitogenic concentrations of growth factors [20••]. These observations suggest that a simultaneous stimulation of Ras by integrins and growth factor receptors is needed to reach a threshold level of MAPK activation required for optimal transcription of Fos. As integrin ligation also activates JNK, it is likely that cell

adhesion will also activate Jun. Fos–Jun heterodimers can then regulate transcription of genes necessary for cell cycle progression [59] (Figure 3).

Recent studies have indicated that cell adhesion is specifically required for the induction of cyclin D1 and for the activation of the cyclin E–cdk2 (cyclin-dependent kinase 2) complex in early–mid G_1 phase [60••,61••]. Although cyclin D1 is regulated by cell adhesion at both the transcriptional and the translational levels, the effect of cell adhesion on cyclin E–cdk2 activity appears to be indirect and mediated by a downregulation of the cdk inhibitors p21 and p27. These findings suggest several mechanisms by which integrin signaling could regulate the cell cycle. In fact, the cyclin D1 promoter contains an SRE and a c-ets site, both of which are targets of ERK signaling [62]. In addition, it has been recently shown that cell adhesion to the ECM causes activation of $p70^{S6K}$ [63,64], a kinase that may regulate cyclin D1 translation and p27 levels by multiple mechanisms (reviewed in [65]).

Regulation of cell migration

A recent study has provided evidence that the activation of ERK in response to integrin ligation may also play a role in regulating cell migration [66•]. In fact, ERK activation by a constitutively active form of the MAPK kinase MEK1 increases cell migration, while ERK inhibition by antisense oligonucleotides or a specific chemical inhibitor reduces cell migration [66•]. Interestingly, the ability of ERK to stimulate cell migration correlates with the phosphorylation and activation of myosin light chain kinase (MLCK). In addition, *in vitro* assays indicate that ERK can phosphorylate MLCK, and ERK-phosphorylated MLCK displays an enhanced capacity to phosphorylate its physiological substrate, the myosin light chain, in a calmodulin-dependent manner. These findings suggest that ERK may regulate cell migration by stimulating actomyosin contractility.

Multiple integrin-dependent pathways are likely to be involved in protection from apoptotic cell death

Normal cells denied anchorage to an appropriate extracellular matrix undergo apoptosis and this may be a mechanism to insure that cells which are displaced from their natural environment are eliminated. Tumor cells appear to be unusually resistant to this homeostatic mechanism, which may explain their propensity to home within tissues different from their own (see Frisch and Ruoslahti, this issue, pp 701–706).

The ability of the ECM to promote cell survival appears to be mediated by at least two distinct signaling pathways. The studies on the role of Shc in integrin signaling have indicated that, in primary cells, adhesion mediated by integrins not linked to Shc results not only in cell cycle arrest, but also in apoptotic cell death [20••]. In accordance with a critical role of Shc signaling in protection from apoptosis, a dominant-negative form of Shc induces apoptotic death in primary endothelial cells plated on fibronectin in presence of mitogens (KK Wary, FG Giancotti, unpublished data). Although not demonstrated, the integrins that combine with Shc may promote cell survival by elevating the activity of ERK.

Other studies have shown that constitutively active forms of FAK and PI3-K can protect cells from suspension-induced apoptosis [26•,67•]. Although the anti-apoptotic effect of PI3-K has been been shown to be mediated by the serine/threonine kinase Akt, the mechanism by which integrins activate PI3-K remains to be established, but probably involves FAK. Alternatively, as PI3-K is a major target effector of Ras [46•], the activation of PI3-K by integrins could be mediated by the ability of Shc to activate Ras. If the first hypothesis is correct, then one could envision a model in which all integrins activate the FAK–PI-3K–Akt pathway, but only a subset is capable of activating the Shc–Ras–ERK pathway. This would imply that both pathways need to be activated for survival (Figure 3).

ECM-induced exit from the cell cycle may facilitate differentiation

It has been known for long that adhesion to an appropriate ECM is required for differentiation in a number of cell types. The studies on Shc-mediated signaling indicate that ECM recognition by integrins that fail to activate Shc results in cell cycle exit even in the presence of mitogens [20••]. As withdrawal from the cell cycle is a prerequisite for differentiation, it is possible that those integrins which do not activate the Shc pathway may promote differentiation. This hypothesis is consistent with a number of previous observations. For example, in endothelial cells, adhesion to fibronectin, which is mediated by the Shc-linked integrin $\alpha5\beta1$, promotes proliferation [20••], but adhesion to a laminin-rich matrix, which is mediated by the non-Shc-linked $\alpha2\beta1$ integrin, promotes exit from the cell cycle and formation of capillary-like structures [68]. Similarly, myoblasts proliferate on fibronectin but fuse to form myotubes on laminin, and these two opposing functions have been linked to the expression of $\alpha5\beta1$ and $\alpha6\beta1$ integrin, respectively [69•].

The ability of the ECM to promote exit from the cell cycle may be necessary, but is unlikely to be sufficient to induce differentiation. In fact, although primary mammary epithelial cells need to adhere to a basement membrane gel in order to activate transcription of tissue-specific genes, such as β-casein and lactoglobulin, this effect requires simultaneous exposure to lactogenic hormones [70]. A recent study has provided evidence that the lactogenic hormone prolactin promotes the binding of the transcription factor Stat5 to γ-interferon activation site (GAS)-related DNA sequence elements in the β-lactoglobulin promoter in breast epithelial cells plated on laminin, but not on collagen or poly-L-lysine [71].

Taken together, these findings suggest that, although interaction with a specific ECM may be sufficient to promote exit from the cell cycle, complete morphological and functional differentiation is likely to require the integration of signaling pathways activated by both integrins and soluble differentiation factors.

Conclusions and future directions

The studies reviewed above indicate that the integrins that are linked to Shc-mediated signaling cooperate with growth factor receptors to stimulate cell proliferation, while the remaining integrins may passively, or perhaps actively, enable exit from the cell cycle and thereby facilitate cell differentiation. There is already evidence pointing to other signaling differences between integrins [32,33,72,73], and future studies will probably reveal additional integrin-specific pathways.

An important conclusion of many studies is that the signaling pathways activated by growth factor receptors and integrins are extensively interconnected, most importantly at the level of Ras, PI-3K and FAK. In addition, there is evidence that certain integrins form physical complexes with growth factor receptors [74,75•]. Future studies will have to address the molecular mechanisms that regulate the integration and, ultimately, the interpretation of signals from integrins and growth factor receptors. In particular, many signaling intermediates interact with, and receive signals from, various upstream elements as well as bind to a large number of downstream target effectors. It will be interesting to dissect which molecular pathways are really converging and diverging at each one of these nodes in response to signals from the ECM and growth factors.

We have just begun to understand how all these pathways influence cell survival, proliferation, and differentiation. Although some membrane-proximal events have been delineated, it will be important to identify the nuclear events controlled by these pathways and understand how these events in turn control the cell cycle and apoptotic machinery. Finally, an analysis of the molecular mechanisms that allow tumor cells to survive and proliferate in the absence of proper signals from the ECM will be an important area of investigation.

Acknowledgements

I am grateful to many colleagues for sharing their results prior to publication and to members of my laboratory for comments on the manuscript. Research in my laboratory is supported by grants from the National Institutes of Health, the Department of Defense Breast Cancer Program, and the American Heart Association.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Giancotti FG, Mainiero F: **Integrin-mediated adhesion and signaling in tumorigenesis.** *Biochim Biophys Acta* 1994, **1198**:47-64.

2. Adams JC, Watt FM: **Regulation of development and differentiation by extracellular matrix.** *Development* 1993, **117**:1183-1198.

3. Ruoslahti E: **Integrins.** *J Clin Invest* 1991, **87**:1-5.

4. Hynes RO: **Integrins: versatility, modulation and signaling in cell adhesion.** *Cell* 1992, **69**:11-25.

5. Hynes RO: **Targeted mutations in cell adhesion genes: what have we learned from them?** *Dev Biol* 1996, **180**:402-412.

6. Parsons JT: **Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins.** *Curr Opin Cell Biol* 1996, **8**:146-152.

7. Lewis JM, Schwartz MA: **Mapping *in vivo* associations of cytoplasmic proteins with β1 cytoplasmic domain mutants.** *Mol Cell Biol* 1995, **6**:151-160.

8. Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, Guan JL: **Interaction of focal adhesion kinase with cytoskeletal protein talin.** *J Biol Chem* 1995, **270**:16995-16999.

9. Brown MC, Perrotta JA, Turner CE: **Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding.** *J Cell Biol* 1996, **135**:1109-1123.

10. Hildebrand JD, Schaller MD, Parsons JT: **Paxillin, a tyrosine-phosphorylated focal adhesion-associated protein, binds to the carboxy-terminal domains of focal adhesion kinase.** *Mol Cell Biol* 1995, **6**:637-647.

11. Schaller MD, Otey CA, Hildebrand JD, Parsons JT: **Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains.** *J Cell Biol* 1995, **130**:1181-1187.

12. Schlaepfer DD, Hunter T: **Focal adhesion kinase overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interaction with and activation of c-Src.** *J Biol Chem* 1997, **272**:13189-13195.

13. Hotchin NA, Hall A: **The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases.** *J Cell Biol* 1995, **131**:1857-1865.

14. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT: **Autophosphorylation of the focal adhesion kinase directs SH2-dependent binding of pp60^{src}.** *Mol Cell Biol* 1994, **14**:1680-1688.

15. • Richardson A, Parsons JT: **A mechanism for regulation of the focal adhesion-associated protein tyrosine kinase pp125^{FAK}.** *Nature* 1996, **380**:538-540.
This paper shows that elevated levels of FRNK, the autonomously expressed carboxy-terminal domain of focal adhesion kinase (FAK), prevent the activation of FAK by a dominant-negative mechanism, thereby inhibiting tyrosine phosphorylation of paxillin and tensin as well as formation of focal adhesions and spreading on fibronectin.

16. Vuori K, Hirai H, Aizawa S, Ruoslahti E: **Induction of p130^{cas} signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases.** *Mol Cell Biol* 1996, **16**:2606-2613.

17. Schlaepfer DD, Broome MA, Hunter T: **Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130^{cas}, and Nck adaptor proteins.** *Mol Cell Biol* 1997, **17**:1702-1713.

18. Schlaepfer DD, Hunter T: **Evidence for *in vivo* phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases.** *Mol Cell Biol* 1996, **6**:5623-5633.

19. Chen HC, Appeddu PA, Isoda H, Guan JL: **Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase.** *J Biol Chem* 1996, **271**:26329-26334.

20. •• Wary KK, Mainiero F, Isakoff SJ, Marcantonio EE, Giancotti FG: **The adaptor protein Shc couples a class of integrins to the control of cell cycle progression.** *Cell* 1996, **87**:733-743.
This study shows that ligation of the α1β1, α5β1, and αvβ3 integrins induces recruitment and tyrosine phosphorylation of Shc, association of Shc with Grb2, and activation of the Ras–ERK mitogen-activated protein kinase pathway. In contrast, ligation of α2β1, α3β1, and α6β1 integrins does not induce these events. Mutagenesis and dominant-negative inhibition studies indicate that Shc is activated independently of focal adhesion kinase and

this event is both necessary and sufficient for the activation of ERK in response to cell adhesion. Integrin-mediated Shc signaling is shown to be necessary for transcription from the Fos serum-response element, protection from apoptotic death, and progression through the G_1 phase of the cell cycle in response to soluble mitogens.

21. • Lin TH, Aplin AE, Shen Y, Chen Q, Schaller M, Romer L, Aukhil I, Juliano RL: **Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts.** *J Cell Biol* 1997, **136**:1385-1395.

This paper shows that a β1 integrin subunit deletion mutant unable to activate focal adhesion kinase (FAK) can promote ERK activation, while FRNK can inhibit FAK without affecting ERK activation.

22. Owens LV, Xu L, Craven RJ, Dent GA, Weiner TM, Kornberg L, Liu ET, Cance WG: **Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors.** *Cancer Res* 1995, **55**:2752-2755.

23. Ilić D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T, Aizawa S: **Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice.** *Nature* 1995, **377**:539-544.

24. Cary LA, Chang JF, Guan JL: **Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn.** *J Cell Sci* 1996, **109**:1787-1794.

25. Gilmore AP, Romer LH: **Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation.** *Mol Cell Biol* 1996, **7**:1209-1224.

26. • Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY: **Control of adhesion-dependent cell survival by focal adhesion kinase.** *J Cell Biol* 1996, **134**:793-799.

Ovexpression of CD2–focal adhesion kinase (FAK), a membrane-anchored form of FAK which is constitutively active and associated with Src, rescues epithelial cells from suspension-induced apoptosis and induces anchorage-independent growth in MDCK cells.

27. Lewis JM, Baskaran R, Taagepera S, Schwartz MA, Wang YJ: **Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport.** *Proc Natl Acad Sci USA* 1996, **93**:15174-15179.

28. •• Gertler FB, Niebuhr K, Reinhard M, Wehland J, Soriano P: **Mena, a relative of VASP and *Drosophila* Enabled, is implicated in the control of microfilament dynamics.** *Cell* 1996, **87**:227-239.

This paper reports the identification of Mena and Evl, two mammalian proteins that are closely related to the *Drosophila* Abl substrate Enabled. The amino-terminal EVH-1 domain of Mena binds to zyxin and vinculin and is necessary for the incorporation of Mena in focal adhesions, while the central proline-rich segment interacts with profilin and regulates microfilament assembly.

29. • Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S: **Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase.** *Nature* 1996, **379**:91-96.

This paper describes the isolation of integrin-linked kinase cDNA and the initial characterization of the encoded kinase.

30. Radeva G, Petrocelli T, Behrend E, Leung-Hagesteijn C, Filmus J, Slingerland J, Dedhar S: **Overexpression of the integrin linked kinase (ILK) promotes anchorage-independent cell cycle progression.** *J Biol Chem* 1997, **272**:13937-13944.

31. Lindberg FP, Gresham HD, Schwarz E, Brown EJ: **Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in αvβ3-dependent ligand binding.** *J Cell Biol* 1993, **123**:485-496.

32. Chung J, Gao AG, Frazier WA: **Thrombospondin acts via integrin-associated protein to activate the platelet integrin αIIbβ3.** *J Biol Chem* 1997, **272**:14740-14746.

33. Berditchevski F, Zutter MM, Hemler ME: **Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins).** *Mol Biol Cell* 1996, **7**:193-207.

34. Berditchevski F, Tolias KF, Wong K, Carpenter CL, Hemler M: **A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase.** *J Biol Chem* 1997, **272**:2595-2598.

35. Lim L, Manser E, Leung T, Hall C: **Regulation of phosphorylation pathways by p21 GTPases: the p21 ras-related Rho subfamily and its role in phosphorylation signalling pathways.** *FEBS Lett* 1996, **242**:171-185.

36. Chong LD, Traynor-Kaplan A, Bokoch GM, Schwartz MA: **The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells.** *Cell* 1994, **79**:507-513.

37. Ren XD, Bokoch GM, Traynor-Kaplan A, Jenkins GH, Anderson RA, Schwartz MA: **Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells.** *Mol Biol Cell* 1996, **7**:435-442.

38. Renshaw MW, Toksoz D, Schwartz MA: **Involvement of the small GTPase Rho in integrin-mediated activation of mitogen-activated protein kinase.** *J Biol Chem* 1996, **271**:21691-21694.

39. • Maniero F, Murgia C, Wary KK, Curatola AM, Pepe A, Blumemberg M, Westwick JK, Der CJ, Giancotti FG: **The coupling of α6β4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation.** *EMBO J* 1997, **16**:2365-2375.

This study shows that Shc links the α6β4 integrin to the Ras–ERK and Rac–JNK signaling pathways. The stimulation of JNK caused by α6β4 integrin ligation requires the activity of Ras, phosphoinositide 3-kinase, and Rac. Adhesion mediated by α6β4 integrin induces serum response element dependent transcription and progression through the G_1 phase of the cell cycle in primary keratinocytes exposed to epidermal growth factor. In contrast, adhesion mediated by α2β1 integrin, which is unable to activate Shc signaling, does not induce these events.

40. •• Gilmore AP, Burridge K: **Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate.** *Nature* 1996, **381**:531-535.

This study shows that phosphatidylinositol 4,5-bisphosphate dissociates the intramolecular interaction between the head and the tail of vinculin, thereby promoting the binding of the head and tail to talin and actin, respectively.

41. Schafer DA, Cooper JA: **Control of actin assembly at filament ends.** *Annu Rev Cell Dev Biol* 1995, **11**:497-518.

42. • Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K: **Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase.** *Science* 1997, **275**:1308-1311.

This study shows that wild-type Rho kinase stimulates the assembly of focal adhesions and stress fibers, while two dominant-negative forms of the kinase suppress the process.

43. • Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K *et al.*: **Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase).** *Science* 1996, **273**:245-248.

This paper shows that Rho kinase phosphorylates and inactivates myosin phosphatase, thereby elevating the amounts of phosphorylated myosin in cells.

44. Chrzanowska-Wodnicka M, Burridge K: **Rho-stimulated contractility drives the formation of stress fibers and focal adhesions.** *J Cell Biol* 1996, **133**:1403-1415.

45. Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM: **Integrin function: molecular hierarchies of cytoskeletal and signalling molecules.** *J Cell Biol* 1995, **131**:791-805.

46. • Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J: **Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras.** *Cell* 1997, **89**:457-467.

Various effector loop mutants of Ras and dominant-negative forms of phosphoinositide 3-kinase (PI-3K) are used to demonstrate that PI-3K is the target effector of Ras that activates Rac and thereby induces reorganization of the actin cytoskeleton. Evidence is provided that oncogenic Ras needs to interact with both PI-3K and Raf to transform fibroblasts.

47. •• Joneson T, McDonough M, Bar-Sagi D, Aelst LV: **RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase.** *Science* 1996, **274**:1374-1376.

This study shows that an activated form of Rac regulates the actin cytoskeleton and promotes cell cycle progression by interacting with target effector(s) distinct from p21(Cdc42/Rac)-activated kinase, which is involved in activation of JNK.

48. •• Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenström P, Bridges T, Chant J, Hall A: **Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade.** *Cell* 1996, **87**:519-529.

This study shows that activated forms of Rac and Cdc42 regulate the actin cytoskeleton and promote cell cycle progression by interacting with target effector(s) distinct from p21(Cdc42/Rac)-activated kinase, which is involved in activation of JNK.

49. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE: **Geometric control of cell life and death.** *Science* 1997, **276**:1425-1428.

50. Mainiero F, Pepe A, Wary KK, Spinardi L, Mohammadi M, Schlessinger J, Giancotti FG: **Signal transduction by the α6β4 integrin: distinct β4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes.** *EMBO J* 1995, **14**:4470-4481.

51. Weiss A, Littman DR: **Signal transduction by lymphocyte antigen receptors.** *Cell* 1994, **76**:263-274.

52. Giancotti FG: **Signal transduction by the α6β4 integrin: charting the path between laminin binding and nuclear events.** *J Cell Sci* 1996, **109**:1165-1172.

53. Spinardi L, Ren YL, Sanders R, Giancotti FG: **The β4 subunit cytoplasmic domain mediates the interaction of α6β4 integrin with the cytoskeleton of hemidesmosomes.** *Mol Cell Biol* 1993, **4**:871-884.

54. Chen Q, Lin TH, Der CJ, Juliano RL: **Integrin-mediated activation of MEK and mitogen activated protein kinase is independent of Ras.** *J Biol Chem* 1996, **271**:18122-18127.

55. Clark EA, Hynes RO: **Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A2 but not for cytoskeletal organization.** *J Biol Chem* 1996, **271**:14814-14818.

56. Pawson T: **Protein modules and signaling networks.** *Nature* 1995, **373**:573-580.

57. •• Lin TH, Chen Q, Howe A, Juliano RL: **Cell anchorage permits efficient signal transduction between Ras and its downstream kinases.** *J Biol Chem* 1997, **272**:8849-8852.

The authors show that platelet-derived growth factor and epidermal growth factor can activate Ras, but not Raf, in suspended fibroblasts.

58. •• King WG, Mattaliano MD, Chan TO, Tsichlis PN, Brugge JS: **PI3-kinase is required for integrin-stimulated AKT and Raf-1/MAP kinase pathway activation.** *Mol Cell Biol* 1997, **17**:4406-4418.

This study shows that cell adhesion activates phosphoinositide 3-kinase (PI-3K), thereby elevating the levels of phosphatidylinositol bisphosphate at the plasma membrane and causing the recruitment and activation of Akt. Inhibition of PI-3K suppresses Raf–ERK signaling without preventing Ras activation in response to cell adhesion.

59. Karin KA: **The regulation of AP-1 activity by mitogen-activated protein kinases.** *J Biol Chem* 1995, **270**:16483-16486.

60. •• Fang F, Orend G, Watanabe N, Hunter T, Ruoslahti E: **Dependence of cyclin E-CDK2 kinase activity on cell anchorage.** *Science* 1996, **271**:499-502.

This study shows that normal fibroblasts deprived of anchorage arrest in late G_1 phase because increased levels of the cyclin-dependent kinase (cdk) inhibitors p21 and p27 combine with cyclin E–cdk2.

61. •• Zhu X, Ohtsubo M, Bohmer R, Roberts JM, Assoian RK: **Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein.** *J Cell Biol* 1996, **133**:391-403.

The authors show that cell adhesion is necessary for transcription and translation of cyclin D1 and downregulation of p21 and p27 in response to mitogens.

62. Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG: **Transforming p21-Ras mutants and c-/ets-2 activate the cyclin D1 promoter through distinguishable regions.** *J Biol Chem* 1996, **270**:23589-23597.

63. Koyama H, Raines EW, Bornfeldt KE, Roberts JM, Ross R: **Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors.** *Cell* 1996, **87**:1069-1078.

64. Malik RK, Parsons JT: **Integrin-dependent activation of the p70 ribosomal S6 kinase signaling pathway.** *J Biol Chem* 1996, **271**:29785-29791.

65. Chou MM, Blenis J: **The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling.** *Curr Opin Cell Biol* 1995, **7**:806-814.

66. • Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresh DA: **Regulation of cell motility by mitogen-activated protein kinase.** *J Cell Biol* 1997, **137**:481-492.

The results of this paper indicate that the mitogen-activated protein kinase ERK regulates the cell motility machinery by phosphorylating and activating myosin light chain kinase.

67. • Khwaja A, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J: **Matrix adhesion and ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway.** *EMBO J* 1997, **16**:2783-2793.

This study implicates phosphoinositide 3-kinase, acting through Akt, as a key mediator of matrix-induced survival of normal epithelial cells and aberrant survival of Ras-transformed epithelial cells.

68. Kubota W, Kleinman HK, Martin GR, Lawley TJ: **Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures.** *J Cell Biol* 1988, **107**:1589-1598.

69. • Sastry SK, Lakonishok M, Thomas DA, Muschler J, Horwitz AF: **Integrin α subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation.** *J Cell Biol* 1996, **133**:169-184.

This paper establishes the concept that α5β1 integrin mediated signaling promotes myoblast proliferation, while α6β1 integrin mediated signaling induces exit of myoblasts from the cell cycle and differentiation to myotubes.

70. Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz APN, Roskelley C, Bissekk MJ: **Laminin mediates tissue-specific gene expression in mammary epithelia.** *J Cell Biol* 1995, **129**:591-603.

71. Streuli CH, Edwards GM, Delcommenne M, Whitelaw CBA, Burdon TG, Schindler C, Watson CJ: **Stat5 as a target for regulation by extracellular matrix.** *J Biol Chem* 1995, **270**:21639-21644.

72. Meredith J, Takada Y, Fornaro M, Languino LR, Schwartz MA: **Inhibition of cell cycle progression by the alternatively spliced integrin β1c.** *Science* 1995, **269**:1570-1572.

73. Dixit RB, Chen A, Chen J, Sheppard D: **Identification of a sequence within the integrin β6 subunit cytoplasmic domain that is required to support the specific effect of αvβ6 on proliferation in three-dimensional culture.** *J Biol Chem* 1996, **271**:25976-25980.

74. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM: **Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors.** *J Cell Biol* 1996, **135**:1633-1642.

75. • Schneller M, Vuori K, Ruoslahti E: **αvβ3 integrin associates with activated insulin and PDGFβ receptors and potentiates the biological activity of PDGF.** *EMBO J* 1997, in press.

The authors show that the activated insulin and PDGFβ receptors combine with αvβ3 integrin. Evidence is also provided that cell adhesion to vitronectin, an αvβ3 ligand, facilitates mitogenesis and chemotaxis in response to PDGFβ.

Cell, Vol. 87, 733–743, November 15, 1996, Copyright ©1996 by Cell Press

The Adaptor Protein Shc Couples a Class of Integrins to the Control of Cell Cycle Progression

Kishore K. Wary,*† Fabrizio Mainiero,*‡ Steven J. Isakoff,§ Eugene E. Marcantonio,‖ and Filippo G. Giancotti*†
*Department of Pathology and Kaplan Cancer Center
§Sackler Institute of Graduate Biomedical Sciences
New York University School of Medicine
New York, New York 10016
‖Department of Pathology
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

We provide evidence that a class of integrins combines with the adaptor Shc and thereby with Grb2. Coimmunoprecipitation and mutagenesis experiments indicate that the recruitment of Shc is specified by the extracellular or transmembrane domain of integrin α subunit and suggest that this process is mediated by caveolin. Mutagenesis and dominant-negative inhibition studies reveal that Shc is necessary and sufficient for activation of the MAP kinase pathway in response to integrin ligation. Mitogens and Shc-activating integrins cooperate to promote transcription from the Fos serum response element and transit through G1. In contrast, adhesion mediated by integrins not linked to Shc results in cell cycle arrest and apoptosis even in presence of mitogens. These findings indicate that the association of specific integrins with Shc regulates cell survival and cell cycle progression.

Introduction

In addition to promoting cell adhesion, the extracellular matrix exerts complex and often divergent effects on cellular behavior. Normal cells require anchorage to the extracellular matrix in order to proliferate, and loss of this growth control mechanism is a hallmark of neoplastic cells (Giancotti and Mainiero, 1994). In a number of settings, however, interaction with the extracellular matrix promotes exit from the cell cycle and differentiation (Lin and Bissell, 1993). Recent results suggest that adhesion to the extracellular matrix may also be required for cell survival (Ruoslahti and Reed, 1994). These diverse activities of the extracellular matrix are likely to be mediated by the ability of integrins to activate intracellular signaling pathways, but the mechanisms involved are incompletely understood.

In vertebrates, cell adhesion to the extracellular matrix is mediated by at least 15 distinct integrins, including nine β_1 and four α_v subunit–containing heterodimers (Ruoslahti, 1991; Hynes, 1992). Individual integrins can recognize several extracellular matrix molecules; conversely, one extracellular matrix molecule usually binds to several integrins. Despite this high degree of apparent redundancy, several integrin gene knockouts cause developmental abnormalities. In particular, mutation of each of the three major fibronectin-binding integrins produces a distinct phenotype, suggesting that integrins with overlapping ligand binding specificities carry out distinct functions (Hynes, 1996). Although differences in ligand binding affinity and association with the cytoskeleton have been demonstrated, the observation that the extracellular matrix can promote either proliferation or growth arrest and differentiation, depending on its composition and the cell type involved (Lin and Bissell, 1993), suggests the existence of signaling differences among integrins.

The intracellular signals elicited by β_1 and α_v integrins resemble those induced by receptor tyrosine kinases, suggesting that tyrosine phosphorylation plays a crucial role in integrin signaling (Clark and Brugge, 1995). Recent studies on integrin signaling have focused on the tyrosine kinase named focal adhesion kinase (FAK) (Schaller and Parsons, 1994). FAK can interact with signaling molecules capable of regulating gene expression (Chen and Guan, 1994; Schlaepfer et al., 1994; Vuori et al., 1996). However, the effects of disrupting FAK by gene targeting or dominant-negative inhibition appear to be limited to the cytoskeleton (Ilić et al., 1995; Richardson and Parsons, 1996). Furthermore, because all β_1 and α_v integrins are able to stimulate FAK, the activation of FAK does not explain the differential effects of extracellular matrix on cellular function.

In this paper, we demonstrate that a subset of β_1 and α_v integrins are linked to the MAP kinase pathway by the adaptor protein Shc, and we provide evidence that this signaling mechanism regulates cell survival and cell cycle progression in response to the extracellular matrix.

Results

Association of β_1 Integrins with Shc and Grb2

Preliminary experiments indicated that cell adhesion to fibronectin results in tyrosine phosphorylation of proteins with apparent molecular masses of 125 kDa, 80 kDa, 52 kDa, and 46 kDa. While the 125 kDa and 80 kDa proteins comigrated with FAK and paxillin, the 52 kDa and 46 kDa proteins displayed a mobility similar to that of the two major isoforms of Shc. Shc is a SH2–phosphotyrosine-binding (PTB) domain adaptor that links tyrosine kinases to Ras signaling by recruiting the Grb2–mSOS complex to the plasma membrane in a tyrosine phosphorylation–dependent manner (Pawson, 1995). To test whether antibody-mediated ligation of β_1 integrins resulted in tyrosine phosphorylation of Shc and association of Shc with Grb2, A431 cells were incubated in suspension with polystyrene beads coated with

†Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, New York 10021.
‡Present address: Dipartimento di Patologia Sperimentale e Medicina, Università La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy.

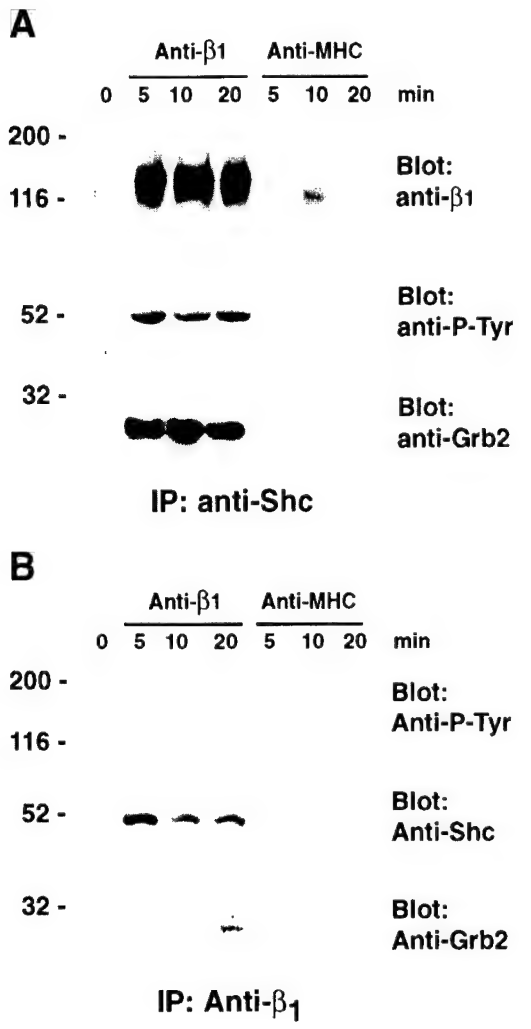


Figure 1. Recruitment of Shc and Grb2 by β_1 Integrins

(A) A431 cells were stimulated in suspension with 4B4 (anti-β_1) or W6.32 (anti-MHC) MAb–coated beads for the indicated times. Equal amounts of proteins were immunoprecipitated with an antiserum reacting predominantly with p52^{shc} and subjected to immunoblotting with anti-β_1 cytoplasmic domain serum, anti-P-Tyr MAb PY20, or anti-Grb2 MAb EL6.

(B) A431 cells were stimulated as above, immunoprecipitated with anti-β_1 MAb TS2/16, and subjected to immunoblotting with anti-P-Tyr MAb PY20, anti-Shc serum, or anti-Grb2 serum.

the anti-β_1 monoclonal antibody (MAb) 4B4 or the control anti–major histocompatibility complex (MHC) MAb W6.32 and immunoprecipitated with an antiserum reacting predominantly with the 52 kDa isoform of Shc. Immunoblotting with anti-phosphotyrosine (anti-P-Tyr) and anti-Grb2 antibodies indicated that cross-linking of β_1 integrins, but not MHC molecules, results in tyrosine phosphorylation of p52^{shc} and association of Shc with Grb2 (Figure 1A).

To examine whether β_1 integrins formed a complex with Shc, anti-Shc immunoprecipitates were subjected to immunoblotting with anti-β_1 antibodies, and anti-β_1 immunoprecipitates were probed by immunoblotting with anti-Shc antibodies. The results indicated that β_1 integrins associate with Shc in response to stimulation

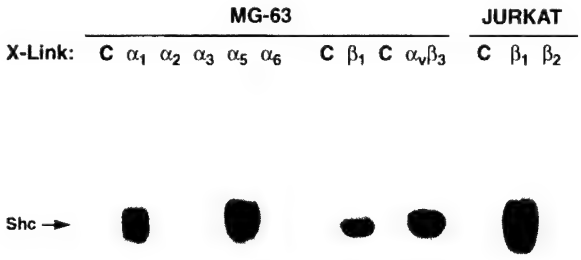


Figure 2. Spectrum of Integrins Linked to Shc

Cells were stimulated in suspension with W6.32 (c), 4B4 (β_1), P4H9 (β_2), LM609 ($\alpha_v\beta_3$), TS2/7 (α_1), PIE6 (α_2), PIB5 (α_3), PID6 (α_5), or GoH3 (α_6) MAb-coated beads, immunoprecipitated with anti-Shc serum, and subjected to immunoblotting with anti-P-Tyr MAb PY20.

with anti-β_1 but not anti-MHC beads (Figure 1). In accordance with the hypothesis that the association of Grb2 with β_1 integrins is predominantly mediated by Shc, we detected a lower amount of Grb2 in anti-β_1 than anti-Shc immunoprecipitates (compare Figure 1A with Figure 1B).

Since the β_1 tail contains two tyrosines (Y783 and Y795) that upon phosphorylation could provide a binding site for the PTB domain of Shc (N-P-X-pY), we tested whether the interaction of β_1 integrins with Shc was mediated by tyrosine phosphorylation of the β_1 tail. However, experiments of immunoblotting with anti-P-Tyr antibodies failed to demonstrate tyrosine phosphorylation of β_1 integrins in cells stimulated with anti-β_1 beads (Figure 1B). In addition, Sepharose beads carrying GST fusion proteins encoding the PTB or SH2 domains of Shc did not bind the β_1 subunit from SDS-denatured extracts of anti-β_1-stimulated cells (data not shown). Taken together, these results are consistent with the hypothesis that β_1 integrins interact with Shc indirectly.

Since Shc potentially links β_1 integrins to Ras signaling, we examined whether ligation of β_1 integrins activated Ras. In vivo Ras–GTP loading experiments indicated that adhesion of primary human keratinocytes to anti-MHC MAb–coated dishes does not result in a significant activation of Ras (18.5% GTP/GTP plus GDP). In contrast, adhesion to anti-β_1 MAb–coated dishes caused activation of Ras to a level (43% GTP/GTP plus GDP) comparable to that induced by treatment with tetradecanoyl phorbol acetate (TPA) and ionomycin (49% GTP/GTP plus GDP). These findings suggest that the recruitment of Shc by β_1 integrins contributes to the activation of Ras in response to cell adhesion.

The Association with Shc Defines a Class of Integrins

To determine the spectrum of integrins linked to Shc, we examined Jurkat cells, which express various β_1 and β_2 integrins, and MG-63 cells, which express the promiscuous integrins $\alpha_v\beta_3$ and $\alpha_3\beta_1$, the collagen/laminin receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, the fibronectin receptor $\alpha_5\beta_1$, and the laminin receptor $\alpha_6\beta_1$. As shown in Figure 2, antibody-mediated cross-linking of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ caused tyrosine phosphorylation of Shc. In contrast,

ligation of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and β_2 integrins did not induce this event. Since the beads coated with anti-α_2, -α_3, and -α_6 MAbs were able to induce tyrosine phosphorylation of FAK (Figure 4C; data not shown), it is unlikely that they mediate inefficient cross-linking or interfere with some other aspect of integrin signaling. Furthermore, beads carrying three different MAbs to the ectodomain of α_6, including two that interfere with the adhesive function of $\alpha_6\beta_1$ and one that does not, failed to induce tyrosine phosphorylation of Shc, while beads coated with three MAbs to α_5, including both adhesion-blocking and -nonblocking reagents, consistently induced this event (data not shown). These results suggest that $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ are unable to combine with Shc and induce its tyrosine phosphorylation. In accordance with this conclusion, coimmunoprecipitation analysis indicated that $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$, associate with Shc upon antibody-mediated cross-linking (see Figures 4A and 4C; data not shown). These findings indicate that integrins with overlapping binding specificity can be distinguished on the basis of their ability to combine with Shc and induce its phosphorylation.

The Association with Shc Is Specified by the Extracellular or Transmembrane Domain of Integrin α Subunit

To define the integrin sequences involved in the recruitment of Shc, we examined NIH 3T3 and Chinese hamster ovary (CHO) cells expressing the recombinant human wild-type and mutant integrin subunits illustrated in Figure 3. Fluorescence-activated cell sorter (FACS) analysis indicated that each mutant integrin subunit was expressed at a level comparable to that of the corresponding recombinant wild-type control. The cells were stimulated with anti-integrin or control MAb–coated beads and subjected to immunoprecipitation and immunoblotting analysis. The results indicated that neither a simultaneous phenylalanine substitution in correspondence of both N-P-X-Y motifs (β_1Y783F-Y795F) nor a deletion of almost the entire cytoplasmic portion of β_1 ($\beta_1\Delta$Cyto) affects the ability of β_1 integrins to associate with Shc (Figure 4A) and induce its phosphorylation (data not shown), confirming the hypothesis that the β_1 cytoplasmic domain does not interact with Shc in a tyrosine phosphorylation–dependent manner and indicating that the majority of the β_1 tail is not required for association with Shc. To our surprise, neither a deletion of the α_1 cytoplasmic domain immediately following the conserved GFFKR box ($\alpha_1\Delta$Cyto) nor a deletion of the entire α_5 tail ($\alpha_5\Delta$Cyto) diminished the ability of $\alpha_1\beta_1$ and $\alpha_5\beta_1$, respectively, to associate with Shc (Figure 4A) and promote its phosphorylation (data not shown). These results indicate that the cytoplasmic domain of α subunit, like the corresponding portion of β subunit, is not required for recruitment and tyrosine phosphorylation of Shc.

To exclude the possibility that each cytoplasmic tail was independently capable of recruiting Shc, 293-T cells were transiently transfected with vectors encoding single-subunit chimeras consisting of the extracellular and transmembrane portion of the interleukin-2 receptor α chain linked to the cytoplasmic tail of either the α_5 (Tac-α_5) or β_1 integrin subunit (Tac-β_1). As shown in Figure 4B, neither cross-linking of Tac-α_5 nor of Tac-β_1 caused recruitment of Shc. Even coclustering of the two chimeras was not sufficient to induce recruitment of Shc. In contrast, ligation of endogenous wild-type β_1 integrins in mock-transfected cells resulted, as expected, in efficient recruitment of Shc. Taken together, the results of this mutational analysis suggest that the recruitment of Shc and its tyrosine phosphorylation are not mediated by the cytoplasmic domains of the integrin α or β subunits.

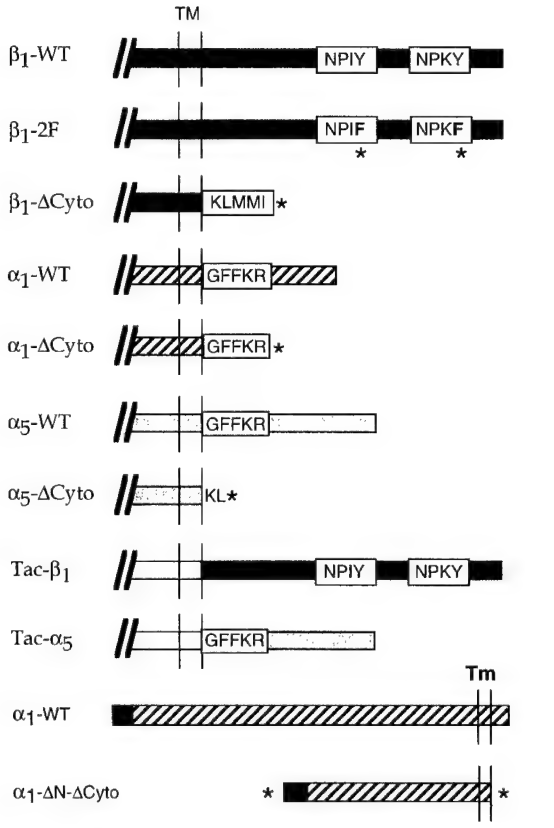


Figure 3. Schematic Representation of Recombinant Wild-Type and Mutant Human Integrin Subunits

Asterisks point to amino acid substitutions and deletions. α_1-WT and α_1-ΔN-ΔCyto are in smaller scale than other mutants.

To explore further the mechanism of recruitment and phosphorylation of Shc in response to integrin stimulation, we examined the properties of a mutant human α_1 subunit that carries a deletion of the N-terminal domain involved in association with β_1 and of the C-terminal tail (see Figure 3). Despite its inability to combine with β_1, this mutant subunit is efficiently exported to the cell surface (Kern et al., 1994). As shown in Figure 4C, ligation of single-chain tailless α_1 caused recruitment and tyrosine phosphorylation of Shc as effectively as stimulation of wild-type $\alpha_1\beta_1$, while ligation of endogenous $\alpha_6\beta_1$ did not induce these events in both transfectants. These results indicate that the association of $\alpha_1\beta_1$, and presumably also $\alpha_5\beta_1$ and $\alpha_v\beta_3$, with Shc is specified by sequences contained in the membrane-proximal portion of the extracellular domain of the α subunit, its transmembrane segment, or both.

Association of Integrins with Caveolin

Because Shc is a cytosolic protein, the results described above suggest that the association of integrins with Shc is mediated by an intermediary membrane component. Caveolin is a 22 kDa transmembrane protein that links a variety of cell-surface receptors lacking a cytoplasmic domain to intracellular signaling pathways (Lisanti et al., 1994). We wished therefore to examine whether caveolin associated with β_1 integrins and Shc. The A431 cells were incubated with anti-MHC or anti-β_1 beads and immunoprecipitated with antibodies to caveolin, Shc, and β_1 integrins. The samples were probed by immunoblotting with antibodies reacting with the β_1 subunit or the three isoforms of Shc. The results revealed that caveolin is constitutively associated with β_1 integrins and forms a complex with Shc upon stimulation with anti-β_1 beads (Figure 5A). The anti-caveolin immunoprecipitates contained similar amounts of the precursor and mature form of β_1, suggesting that β_1 integrins associate with caveolin in the endoplasmic reticulum. To obtain evidence that the association of β_1 integrins with caveolin was not an artifact of cell lysis, we performed immunofluorescent staining of primary human fibroblasts. The results indicated that a significant fraction of caveolin is not diffusely distributed at the cell surface, but coaligns with β_1 integrins, fibronectin fibrils, and actin stress fibers at extracellular matrix contact sites (data not shown). These findings indicate that β_1 integrins associate with caveolin.

To explore further the hypothesis that the recruitment of Shc by integrins was mediated by caveolin, we examined whether the association with caveolin and the recruitment of Shc required the same integrin sequences. NIH 3T3 cells expressing either the human recombinant wild-type α_1 subunit or single-chain tailless α_1 subunit were immunoprecipitated with the anti–human α_1 MAb TS2/7 or the control anti-MHC MAb W6.32, and the samples were subjected to immunoblotting with anti-caveolin antibodies. The results showed that the wild-type $\alpha_1\beta_1$ subunit and the single-chain tailless α_1 subunit associate to similar extent with the 22 kDa isoform of caveolin (Figure 5B). Prolonged exposure of the autoradiograph indicated that they both also form a complex with the 23 kDa minor isoform of caveolin. These results indicate that the association of $\alpha_1\beta_1$ with caveolin is specified by sequences contained in the membrane-proximal portion of the extracellular domain of the α subunit, its transmembrane segment, or both, i.e., the same sequences involved in the recruitment of Shc. Taken together, these findings identify caveolin as a potential mediator of the interaction between integrins and Shc.

Role of Shc in MAP Kinase Activation by Integrins

Since the recruitment of Shc is mediated by the integrin α subunit, while the activation of FAK requires the β subunit, we sought to examine whether the activation of Shc and FAK were independent phenomena. Experiments of immunoprecipitation with anti-FAK antibodies followed by immunoblotting with anti-P-Tyr antibodies indicated that ligation of single-chain tailless α_1 does

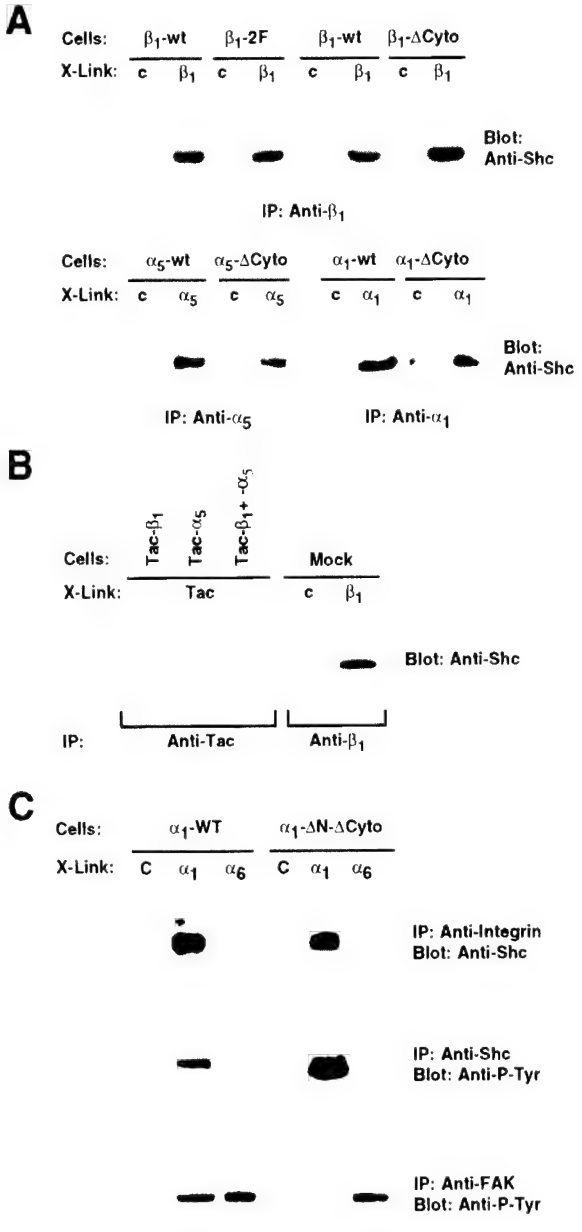


Figure 4. The Recruitment of Shc Is Specified by the Extracellular Domain of the α Subunit, Its Transmembrane Domain, or Both

(A) NIH 3T3-β_1-WT, NIH 3T3-β_1-2F, CHO-β_1-WT, and CHO-β_1-ΔCyto cells were stimulated with W6.32 (c) or 4B4 (β_1) MAb–coated beads, immunoprecipitated with anti-β_1 MAb TS2/16, and probed by immunoblotting with anti-Shc serum. CHO-α_5-WT, CHO-α_5-ΔCyto, NIH 3T3-α_1-WT, and NIH 3T3-α_1-ΔCyto cells were stimulated with W6.32 (c), P1D6 (α_5), or TS2/7 (α_1) MAb–coated beads, immunoprecipitated with MAb P1D6 or TS2/7, and probed by immunoblotting with anti-Shc serum.

(B) 293-T cells were transfected with Tac-β_1 and Tac-α_5, either separately or in combination, stimulated with anti-Tac MAb 4E3, and immunoprecipitated with the same antibody. Mock-transfected cells were stimulated with W6.32 (c) or 4B4 (β_1) MAbs and immunoprecipitated with MAb 4B4. Samples were probed with anti-Shc serum.

(C) After stimulation with W6.32 (c), TS2/7 (α_1), or GoH3 (α_6) MAbs, NIH 3T3-α_1-WT and NIH 3T3-α_1-ΔN-ΔCyto cells were immunoprecipitated with the same antibodies and probed with anti-Shc serum,

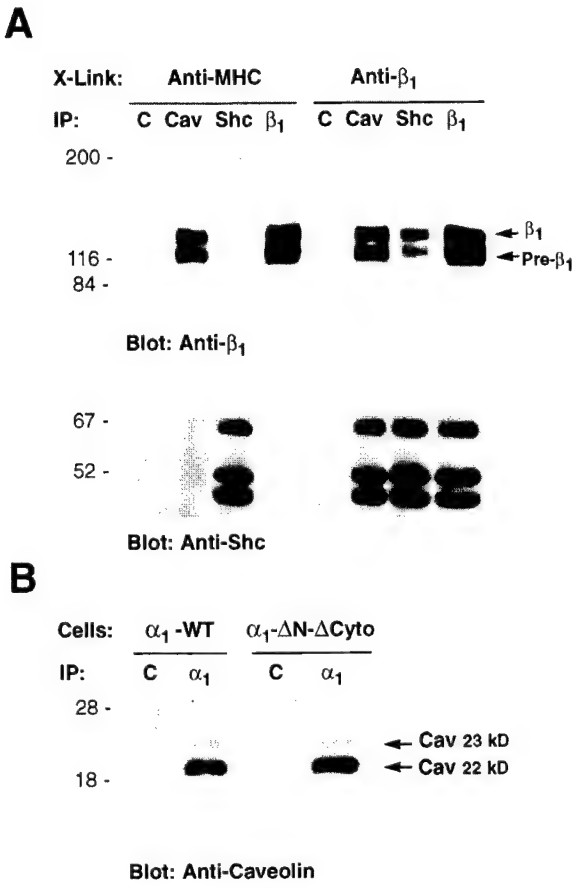


Figure 5. Association of β_1 Integrins with Caveolin

(A) A431 cells were stimulated with W6.32 (anti-MHC) or 4B4 (anti-β_1) MAbs for 10 min, immunoprecipitated with MAb W6.32 (c), C060 (Cav), clone 8 (Shc), or TS2/16 (β_1), and probed with anti-β_1 cytoplasmic domain serum or affinity-purified antibodies to Shc.

(B) NIH 3T3-α_1-WT and NIH 3T3 α_1-ΔN-ΔCyto cells were immunoprecipitated with W6.32 (c) or TS2/7 (α_1) MAbs followed by immunoblotting with anti-caveolin antibodies.

not induce tyrosine phosphorylation of FAK, while stimulation of wild-type $\alpha_1\beta_1$ and endogenous $\alpha_6\beta_1$ induces significant tyrosine phosphorylation of FAK (see Figure 4C). Together with the data of Figure 2, these results indicate that the single-chain tailless α_1 subunit can recruit Shc, but not activate FAK; wild-type $\alpha_6\beta_1$ can activate FAK, but not recruit Shc; and $\alpha_1\beta_1$ can both recruit Shc and activate FAK. Thus, the recruitment and tyrosine phosphorylation of Shc and the activation of FAK are separable phenomena.

To assess the relative contribution of Shc and FAK to the activation of the Ras–mitogen-activated protein (MAP) kinase pathway by integrins, we examined the ability of various integrins to activate the MAP kinase Erk2. NIH 3T3 cells expressing the single-chain tailless α_1 subunit were transiently transfected with a hemagglutinin (HA)-tagged Erk2 vector, stimulated with beads coated with fibronectin, various anti-integrin MAbs, or fetal calf serum (FCS), and subjected to immune complex kinase assays. As shown in Figure 6A, antibody-mediated ligation of single-chain tailless α_1 or endogenous $\alpha_5\beta_1$ activated MAP kinase to a level similar to that induced by fibronectin and serum. In contrast, cross-linking of endogenous $\alpha_6\beta_1$ did not induce this activity. Since the single-chain tailless α_1 subunit is capable of recruiting Shc, but not activating FAK, while cross-linking of $\alpha_6\beta_1$ can stimulate FAK, but does not induce association with Shc, these results suggest that Shc, and not FAK, plays a crucial role in the activation of MAP kinase by integrins.

The role of Shc in the activation of MAP kinase by integrins was further examined by testing the effect of wild-type and dominant-negative Shc (Y317F). This mutant can exert a dominant-negative effect because it is able to combine with tyrosine-phosphorylated proteins but not with Grb2 (Salcini et al., 1994; Chen et al., 1996). The introduction of wild-type Shc led to a dose-dependent increase in Erk2 activation in response to ligation of wild-type $\alpha_1\beta_1$ or single-chain tailless α_1. Conversely, dominant-negative Shc suppressed the activation of MAP kinase in response to ligation of both molecules as effectively as dominant-negative Ras (N17) (Figure 6B). Since full activation of FAK may require cell spreading on the extracellular matrix and cytoskeletal organization, we also examined the effect of dominant-negative Shc on MAP kinase activation in NIH 3T3 cells freshly plated on fibronectin. The introduction of dominant negative Shc and dominant-negative Ras prevented the activation of Erk2 in response to cell adhesion to fibronectin (Figure 6C). We concluded that Shc plays a crucial role in the activation of the Ras–MAP kinase pathway by integrins.

The observation that only a subset of integrins can combine with Shc suggests that cell adhesion to the extracellular matrix may or may not activate Ras signaling, depending on the repertoire of integrins involved. To test this hypothesis, we examined primary human umbilical vein endothelial cells (HUVECs), because it is known that they adhere to fibronectin primarily through $\alpha_5\beta_1$ (Conforti et al., 1989), to vitronectin through $\alpha_v\beta_3$ (Cheresh, 1987), and to laminin 1 and laminin 4 through $\alpha_2\beta_1$ (Languino et al., 1989). In addition, FACS analysis indicated that they do not express the $\alpha_1\beta_1$ collagen/laminin receptor. The HUVECs were either kept in suspension or plated on dishes coated with 10 μg/ml poly-L-lysine, fibronectin, vitronectin, laminin 1, or laminin 4. Under these conditions, the cells adhered to and spread on each extracellular matrix substratum to the same extent. As shown in Figure 6D, adhesion of HUVECs to fibronectin and vitronectin resulted in tyrosine phosphorylation of the three isoforms of Shc, association of Shc with Grb2, and activation of MAP kinase. In contrast, adhesion to laminin 1 or laminin 4 did not induce these events. These results are in accordance with the observation that $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, can combine with Shc and activate the Ras–MAP kinase pathway and indicate that the extracellular matrix may have selective

immunoprecipitated with anti-Shc serum and probed with a mixture of anti-P-Tyr MAbs PY20 and 4G10, or immunoprecipitated with anti-FAK polyclonal antibodies and probed with a mixture of anti-P-Tyr MAbs PY-20 and 4G10.

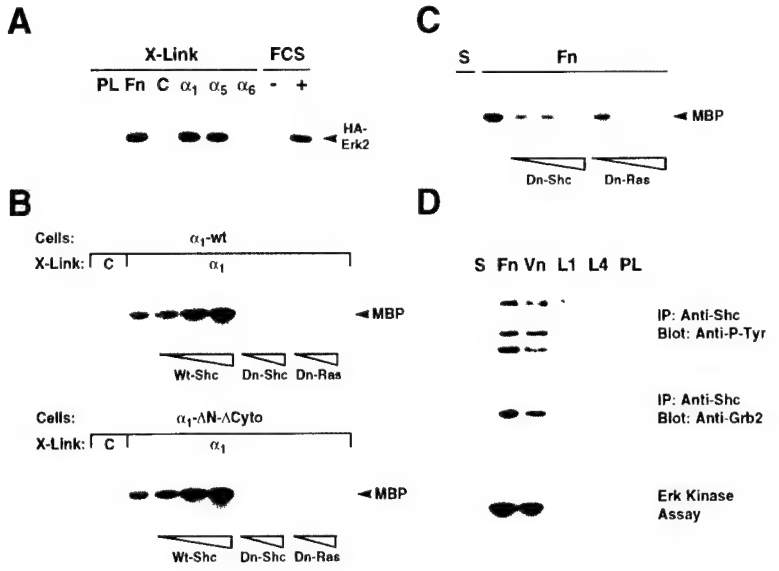


Figure 6. Role of Shc in Activation of MAP Kinase by Integrins

(A) NIH 3T3-α_1-ΔN-ΔCyto cells were transiently transfected with HA-tagged Erk2 plasmid, growth factor–starved, and stimulated in suspension with beads coated with poly-L-lysine (PL), fibronectin (Fn), or MAbs W6.32 (c), TS2/7(α_1), 5H10-27 (α_5), and GoH3 (α_6). As a control, the cells were stimulated with 10% FCS (plus) or left untreated (minus). HA–Erk2 was immunoprecipitated and subjected to in vitro kinase assay.

(B) NIH 3T3-α_1-WT and NIH 3T3-α_1-ΔN-ΔCyto cells were transiently transfected with 3 μg of Erk2-HA plasmid alone or in combination with 2.5, 5.0, and 10 μg of wild-type Shc plasmid (WT-Shc), 2.5 and 5.0 μg of dominant negative Shc plasmid (Dn-Shc), and 2.5 and 5.0 μg of dominant-negative Ras plasmid (Dn-Ras). The cells were stimulated with MAbs W6.32 (c) or TS2/7 (α_1). Immunoprecipitated HA–Erk2 was subjected to in vitro kinase assay with MBP as a substrate. Transfection efficiencies were verified by immunoblotting aliquots of total proteins with anti-HA antibodies.

(C) NIH 3T3 cells were transiently transfected with 3 μg of HA–Erk2 plasmid alone or in combination with 2.5, 5.0, and 10 μg of dominant-negative Shc (Dn-Shc) or Ras plasmid (Dn-Ras). After starvation, the cells were either kept in suspension or plated on fibronectin-coated dishes. HA–Erk2 was immunoprecipitated and subjected to in vitro kinase assay with MBP as a substrate. Transfection efficiencies were verified as above.

(D) HUVECs were growth factor–starved and either kept in suspension or plated on dishes coated with poly-L-lysine (PL), fibronectin (Fn), vitronectin (Vn), laminin 1 (L1), or laminin 4 (L4). Lysates were immunoprecipitated with anti-Shc MAb clone 8 followed by immunoblotting with anti-P-Tyr MAb RC-20 (top) or anti-Grb2 serum (middle). HUVECs were transiently transfected with HA–Erk2 plasmid prior to plating on the various substrata. HA–Erk2 was immunoprecipitated and subjected to in vitro kinase assay with MBP as a substrate (bottom). Transfection efficiencies were verified as above.

effects on intracellular signaling depending on the integrins to which it binds.

Adhesion Mediated by Integrins Linked to Shc Promotes Transcription from the Fos Serum Response Element and Cell Cycle Progression

We next examined whether the coupling of specific integrins to Shc played a role in the control of immediate-early gene expression. Since Erk1 and Erk2 regulate transcription from the Fos serum response element (SRE) by phosphorylating the ternary complex factors Elk-1 and SAP-1 (Treisman, 1995), we examined the effect of integrin ligation on the Fos SRE. HUVECs were transiently transfected with a vector containing the Fos SRE promoter element linked to the luciferase reporter gene and plated on dishes coated with extracellular matrix proteins or poly-L-lysine. The results of the luciferase assay indicated that adhesion to fibronectin and vitronectin causes elevation of Fos SRE–dependent transcription in HUVECs, while adhesion to poly-L-lysine, laminin 1, or laminin 4 does not (Figure 7A). This result indicates that ligation of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, is sufficient to promote transcription from the Fos SRE. Interestingly, treatment with basic fibroblast growth factor (bFGF) induced a significant elevation of Fos SRE activity in HUVECs plated on fibronectin and vitronectin, but caused a remarkably modest effect in cells adhering to poly-L-lysine, laminin 1, or laminin 4 (Figure 7A), suggesting that ligation of integrins linked to Shc, such as $\alpha_5\beta_1$ or $\alpha_v\beta_3$, is required for induction of Fos SRE–dependent transcription in cells exposed to mitogenic growth factors.

To examine whether the integrins linked to Shc played a role in cell cycle progression, HUVECs were syncronized in G0 by growth factor starvation and then plated in presence of mitogens on plastic wells coated with extracellular matrix proteins or poly-L-lysine. Entry into the S phase was examined by 5′-bromo-2′-deoxy-uridine (BrdU) incorporation and anti-BrdU staining. The large majority of HUVECs adhering to fibronectin (96.9% ± 2.7%) and vitronectin (96.2% ± 2.1%) entered the S phase during the 24 hr of the assay. In contrast, only a modest percentage of cells plated on laminin 1 (11.8% ± 4.4%), laminin 4 (21.2% ± 9%), or poly-L-lysine (0.8% ± 1.3%) entered into S phase under the same conditions (Figure 7B). Since this percentage did not increase over an additional 24-hr period, it is unlikely that adhesion to poly-L-lysine or laminins simply delays entry into S phase. In addition, because the HUVECs acquired and maintained a well-spread morphology on laminins (Figure 7B), their inability to enter into S phase on these substrata is not the result of insufficient spreading. Interestingly, if the endothelial cells were plated on poly-L-lysine or laminins in the presence of 10% FCS, which contains fibronectin and vitronectin, they progressed normally through G1 and entered the S phase. The results of these experiments indicate that attachment and spreading on the extracellular matrix are not sufficient for progression of primary cells through G1 in

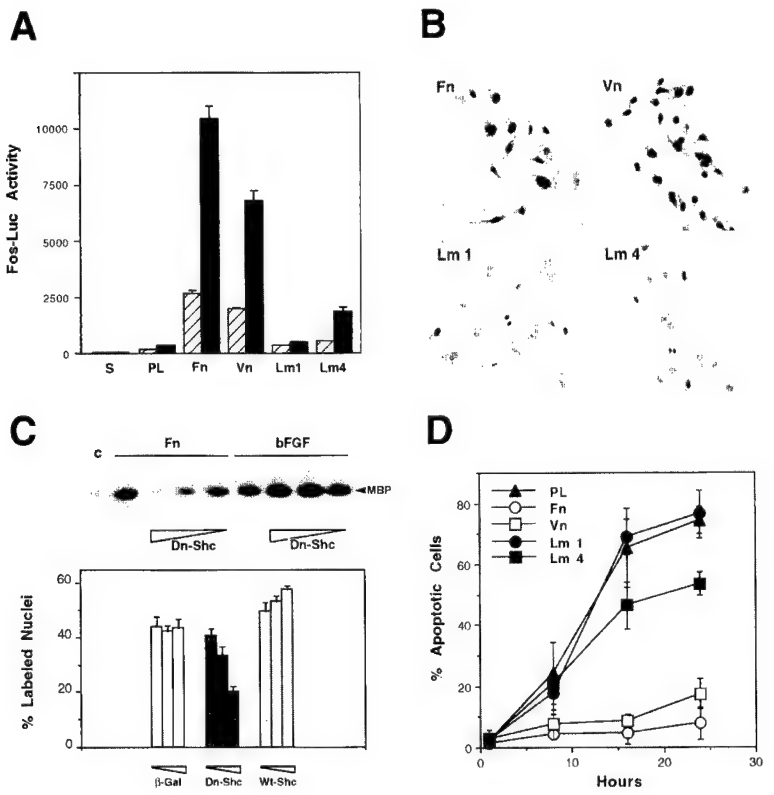


Figure 7. Control of Fos-SRE-Dependent Transcription, Cell Survival, and Cell Cycle Progression by the Extracellular Matrix

(A) HUVECs were transiently transfected with Fos-SRE-Luc plasmid. After growth factor starvation, the cells were either kept in suspension (S) or plated onto dishes coated with 10 μg/ml poly-L-lysine (PL), fibronectin (Fn), laminin 1 (Lm1), or laminin 4 (Lm4) for 20 min. The cells were then either left untreated (hatched bars) or exposed to 10 ng/ml bFGF and 1 μg/ml heparin (closed bars) for 5 min. Cell lysates containing equal amounts of total proteins were subjected to luciferase assay. Values are expressed in arbitrary units.

(B) HUVECs were growth factor–starved and plated for 24 hr in defined medium containing 10 μM BrdU onto wells coated with 10 μg/ml fibronectin (Fn), vitronectin (Vn), laminin 1 (Lm1), or laminin 4 (Lm4). After immunostaining with anti-BrdU monoclonal antibodies and alkaline phosphatase–conjugated secondary antibodies, the cells were lightly counterstained with hematoxylin.

(C) HUVECs were transiently transfected with 3 μg of HA–Erk2 plasmid alone or in combination with 10, 5, and 2.5 μg of plasmid encoding dominant-negative Shc (Dn-Shc). After growth factor starvation, the cells were detached and kept in suspension (c) or plated on fibronectin-coated dishes (Fn), or they were not detached but were treated with 25 ng/ml bFGF and 1 μg/ml heparin for 5 min (bFGF). Transfection efficiencies were verified by immunoblotting with anti-HA MAb. Immunoprecipitated HA–Erk2 was subjected to in vitro kinase assay with MBP as a substrate (top). HUVECs were transiently transfected with 10, 5, and 2.5 μg of plasmids encoding HA-tagged β-galactosidase (β-Gal), FLAG-tagged dominant negative Shc (Dn-Shc), or FLAG-tagged wild-type Shc (WT-Shc). The percentage of transfected cells entering into S was determined as described in Experimental Procedures. The diagram shows the mean value and standard deviation from triplicate samples (bottom).

(D) G0-synchronized HUVECs were plated on wells coated with 10 μg/ml poly-L-lysine (PL), fibronectin (Fn), laminin 1 (Lm1), or laminin 4 (Lm4). Adherent cells were incubated in defined medium for the indicated times. At each time point, attached and unattached cells were combined and stained in suspension with Hoechst dye. The percentage of apoptotic nuclei was determined by scoring at least 500 cells from five different microscopic fields. Diagram indicates the mean and standard deviation from triplicate samples.

response to mitogens, but that this process requires ligation of integrins, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which are coupled to Ras signaling by Shc.

To examine further the role of integrin signaling in cell proliferation, we examined the effect of dominant-negative or wild-type Shc on HUVEC progression through G1. Varying concentrations of vectors encoding FLAG-tagged dominant-negative or wild-type Shc were transfected in HUVECs. After growth factor starvation and restimulation with mitogens, the entry into S phase of cells expressing FLAG-tagged Shc molecules was monitored by double immunofluorescent staining with anti-FLAG and anti-BrdU antibodies. As a control, HUVECs were transfected with varying doses of a vector encoding HA-tagged β-galactosidase, and the entry into S phase of these cells was evaluated by double immunofluorescent staining with anti-HA and anti-BrdU antibodies. As shown in Figure 7C (bottom), the introduction of dominant-negative Shc caused a dose-dependent inhibition of HUVEC entry into S phase. Conversely, wild-type Shc accelerated entry into S, although to a limited extent. The inhibition of cell cycle progression caused by dominant-negative Shc was significant but incomplete, perhaps because transient transfection does not allow high-level expression of the inhibitory molecule over the relatively prolonged duration of G1. It is unlikely that dominant-negative Shc interferes with mitogen stimulation of HUVECs, because it affected the activation of Erk2 by fibronectin, but not by bFGF (Figure 7C, top). These results are consistent with the observation that the FGF receptor 1 is linked to Ras by both Shc-dependent and Shc-independent mechanisms (Mohammadi et al., 1996) and support the notion that the coupling of integrins to Ras signaling mediated by Shc regulates cell cycle progression.

Adhesion Mediated by Integrins Not Linked to Shc Results in Apoptotic Death

A large fraction of endothelial cells, which initially adhered on poly-L-lysine or laminins, subsequently lost phase density and detached, despite being exposed to optimal concentrations of growth factors. We therefore examined whether ligation of integrins linked to Shc protected endothelial cells from apoptosis. HUVECs were plated at low density in the presence of mitogens on dishes coated with extracellular matrix molecules or poly-L-lysine. At various times after plating, adherent

and floating cells were removed, combined, and examined for features of apoptotic death by staining with Hoechst dye. As shown in Figure 7D, only a small number of HUVECs plated on fibronectin or vitronectin underwent apoptosis during the 24 hr of the assay. In contrast, the majority of cells plated on poly-L-lysine or laminins became apoptotic by the end of the assay. Direct staining of cells adhering to poly-L-lysine or laminins revealed that most of them had become apoptotic by 12 hr, suggesting that detachment follows apoptosis and not vice versa. These findings suggest that ligation of integrins linked to Shc is required for endothelial cell survival.

Discussion

The results of this study indicate that a subset of β_1 integrins and $\alpha_v\beta_3$ are linked to Ras signaling and immediate-early gene expression by the adaptor protein Shc. Ligation of integrins linked to Shc enables primary endothelial cells to progress through G1 in response to mitogens, whereas ligation of other integrins, under the same conditions, results in exit from the cell cycle. On the basis of these findings and the ability of dominant-negative Shc to inhibit cell cycle progression without affecting mitogen signaling, we propose that the association of specific integrins with Shc controls cell cycle progression in response to the extracellular matrix. Since exit from the cell cycle is a prerequisite for cell differentiation, our results may also explain why interaction with the extracellular matrix in some settings promotes differentiation.

Shc is a SH2-PTB domain adaptor protein that links various tyrosine-phosphorylated signal transducers to Ras (Pawson, 1995). We have previously shown that ligation of the $\alpha_6\beta_4$ integrin, which is associated with a tyrosine kinase, causes phosphorylation of the β_4 tail and direct recruitment of Shc (Mainiero et al., 1995). In this study, we provide evidence that specific β_1 integrins and $\alpha_v\beta_3$ also combine with Shc, but by a distinct and novel mechanism. In this case, the recruitment of Shc is mediated by the membrane-proximal portion of the extracellular domain of the integrin α subunit, its transmembrane segment, or both. Coimmunoprecipitation experiments revealed that this region of the α subunit interacts constitutively with caveolin, a two-pass transmembrane adaptor involved in linking a variety of cell-surface receptors to intracellular signaling pathways (Lisanti et al., 1994), and indicated that Shc associates with caveolin in response to integrin ligation. Although a definitive demonstration that the recruitment of Shc to β_1 integrins and $\alpha_v\beta_3$ is mediated by caveolin will require further biochemical and mutational analysis, this model is intriguing, because caveolin is phosphorylated on tyrosine in cells transformed by v-Src (Glenney and Soppet, 1992) and has also been shown to interact with c-Fyn (Corley Mastick et al., 1995). Thus, caveolin may provide both the adaptor and the tyrosine kinase necessary for the recruitment and tyrosine phosphorylation of Shc in response to integrin ligation.

There are several reasons to believe that the association of specific integrins with Shc mediates activation of the MAP kinase pathway and transcription of Fos in response to the extracellular matrix. First, ligation of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$, which are linked to Shc, results in MAP kinase activation, but ligation of other integrins does not produce this effect, despite stimulating FAK. Second, cross-linking of the single-chain tailless α_1 subunit causes recruitment and tyrosine phosphorylation of Shc and activation of MAP kinase without inducing FAK activation. Third, a dominant-negative version of Shc suppresses MAP kinase activation in response to integrin ligation. Fourth, while adhesion of endothelial cells to fibronectin and vitronectin, which is mediated by the Shc-linked $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, activates MAP kinase and induces transcription from the Fos SRE, their interaction with laminin 1 and laminin 4, which is mediated by $\alpha_2\beta_1$, does not cause these effects. Taken together, these results indicate that Shc plays a crucial role in the activation of the Ras–MAP kinase pathway and Fos gene expression in response to the extracellular matrix.

Our findings suggest that the association of specific integrins with Shc regulates cell cycle progression in normal cells. In fact, engagement of integrins linked to Shc activates SRE-dependent transcription and promotes progression through G1 in response to growth factors. In contrast, ligation of other integrins results in cell cycle arrest even in the presence of otherwise mitogenic concentrations of growth factors. Normal cells require a signal from the extracellular matrix in order to proliferate. Recent studies have indicated that cell adhesion is necessary for the induction of cyclin D_1 and activation of the cyclin E–cdk2 complex in early to mid-G1 (Fang et al., 1996; Zhu et al., 1996), suggesting that mitogen- and cell adhesion–dependent signals are integrated prior to the induction of cyclin D_1. In accordance with this hypothesis, our findings suggest that integrin- and growth factor–dependent signals converge on Ras.

It is likely that a simultaneous stimulation of Ras by integrins and growth factor receptors is needed to reach the threshold level of MAP kinase activation required for optimal transcription of immediate-early response genes. Since mitogenic growth factors induce a rapid and short-lived stimulation of MAP kinase, while cell adhesion produces a long-lasting activation of the enzyme (Zhu and Assoian, 1995), the two stimuli may also cooperate kinetically. This model is consistent with the observation that overexpression of Shc (Pelicci et al., 1992) and constitutive activation of MAP kinase (Cowley et al., 1994) lead to anchorage-independent cell growth. Furthermore, since most dominant oncogenes transform cells by activating the Ras–MAP kinase pathway, it also explains why most transformed cells display anchorage-independent growth.

Recent studies have revealed that normal cells denied anchorage to the extracellular matrix undergo apoptosis (Ruoslahti and Reed, 1994). The findings of this study suggest that the ability of the extracellular matrix to promote cell survival is mediated by the coupling of specific integrins to Shc. Since inhibition of MAP kinase or activation of Jun kinase cause apoptosis (Xia et al., 1995), it is possible that the integrins that combine with Shc promote cell survival by elevating the activity of MAP kinase, thereby increasing the ratio of MAP kinase

to Jun kinase activity. The anti-apoptotic function of integrins linked to Shc is consistent with the results of previous studies. For example, antibodies and synthetic peptides, which interfere with the adhesive function of $\alpha_v\beta_3$, induce endothelial cell apoptosis and thereby blunt angiogenesis in vivo (Brooks et al., 1994). We suggest, on the basis of our results, that these reagents do not trigger intracellular signals that lead to apoptosis, but rather prevent the activation of Shc signaling caused by $\alpha_v\beta_3$ ligation. The ability of fibronectin to protect CHO cells from apoptosis in response to serum withdrawal (Zhang et al., 1995) and that of basement membrane components, but not type I collagen, to promote survival of breast epithelial cells (Pullan et al., 1996) may also depend on the engagement of specific integrins linked to Shc.

We have observed that intercellular contact can rescue primary endothelial cells plated on laminins from apoptotic death (unpublished data). It has been reported that cell-to-cell contact exerts a similar protective effect in primary breast epithelial cells (Pullan et al., 1996). Although the mechanism by which physical contact between cells protects primary cells from apoptosis is not known, these observations suggest that multiple positional signals contribute to cell survival in vivo. It can be envisioned that in vivo these signals promote the survival of those cells that have, as part of their natural life cycle, lost contact with a matrix capable of activating the Shc pathway. This mechanism may ensure that only cells displaced from their natural environment are eliminated.

What is the fate of those cells that survive despite lacking the Shc signal? The observation that extracellular matrix recognition by integrins that fail to activate Shc results in cell cycle exit even in the presence of mitogens suggests that these cells may be induced to differentiate. It is known that withdrawal from the cell cycle is a prerequisite for differentiation, and several mechanisms ensure that proliferation and differentiation are mutually exclusive. For example, active cyclin D_1–cdk complexes suppress MyoD function in proliferating myoblasts, thereby preventing the expression of muscle-specific genes (Skapek et al., 1995). Conversely, MyoD may maintain the G0 arrest of differentiated skeletal muscle by acting on Rb (Gu et al., 1993). These observations suggest that those integrins that do not activate the Shc pathway may promote differentiation primarily because they do not induce cyclin D_1–cdk levels sufficient to block the function of transcription factors involved in differentiation.

The existence of two classes of integrins with distinct signaling properties explains a number of previous observations. In various cell types, interaction with fibronectin promotes proliferation and inhibits differentiation, while adhesion to laminin promotes cell cycle withdrawal and morphological and functional differentiation. For example, endothelial cells plated on fibronectin proliferate, but on a laminin-rich matrix they cease growing and rapidly form capillary-like structures (Kubota et al., 1988). Similarly, myoblasts proliferate on fibronectin, but fuse to form myotubes on laminin (von der Mark and Ocalan, 1989). These two opposing functions have been linked to the expression of $\alpha_5\beta_1$ and $\alpha_6\beta_1$, respectively (Sastry et al., 1996). Furthermore, recent studies have indicated that the binding of fibronectin to $\alpha_5\beta_1$, but not $\alpha_4\beta_1$, results in induction of the collagenase gene in synovial fibroblasts, suggesting that the same matrix molecule may or may not induce gene expression depending on the integrin to which it binds (Huhtala et al., 1995). Taken together, our findings suggest that the differential responses of a given cell type to extracellular matrices of different composition and of different cell types to the same extracellular matrix protein may all depend on the ability of a class of integrins to activate Shc signaling.

Experimental Procedures

Antibodies and Extracellular Matrix Molecules

The MAbs TS2/7, FW-14–14–15, and TS2/16 were obtained from the American Type Culture Collection, P1E6, P1B5, P1D6, and P4H9 from GIBCO BRL, 5H10–27 from Pharmingen, GoH3 from Immunotech, 4B4 from Coulter, 4E3 and 12CA5 from Boehringer, M2 from Eastman-Kodak, 4G10 from Upstate Biotechnology, Incorporated, and PY20, RC-20, clone 8, and C060 from Transduction Laboratories. The anti-β_1 cytoplasmic domain serum and serum 410, which reacts predominantly with the 52 kDa isoform of Shc, were described (Giancotti and Ruoslahti, 1990; Mainiero et al., 1995). The MAbs AIIB2 and BIE5 were provided by C. Damsky, LM609 by D. Cheresh, and 135–13C by S. Kennel. The anti-P-Tyr serum 72 and anti-Grb2 MAb EL-6 were generated in the laboratory of J. Schlessinger. Affinity-purified rabbit antibodies to Shc and caveolin were from Upstate Biotechnology and Transduction Laboratories, respectively. Anti-Grb2 and anti-Erk2 sera were from Santa Cruz Biotechnology. The anti-FAK peptide serum was generated in the laboratory of G. Tarone. Human fibronectin, vitronectin, laminin 4 (placental merosin; Spinardi et al., 1995), and mouse laminin 1 were purchased from GIBCO BRL.

Cell Lines, Constructs, and Transfections

CHO cells expressing β_1-WT and β_1-ΔCyto were generated in the laboratory of G. Tarone (University of Torino, Turin, Italy). CHO cells expressing α_5-WT or α_5-ΔCyto and NIH 3T3 cells expressing α_1-WT, α_1-ΔCyto, or α_1-ΔN-ΔCyto were previously described (Bauer et al., 1993; Briesewitz et al., 1993; Kern et al., 1994). NIH 3T3 cells expressing β_1-WT and β_1-2F were generated as previously described (Giancotti et al., 1994). Transient transfection of vectors encoding Tac-α_5Cyto and Tac-β_1Cyto (LaFlamme et al., 1992) in 293-T cells was also done as described (Giancotti et al., 1994). The Fos(SRE)-Luc reporter plasmid (from J. Schlessinger) and vectors encoding HA-tagged Erk2, HA-tagged β-galactosidase, FLAG-tagged and untagged wild-type or dominant-negative $p52^{shc}$ (Y317F), and dominant negative Ras (N17) (from E. Scolnik, New York University School of Medicine) were transiently transfected in NIH 3T3 cells by the lipofectamine method and in HUVECs by the lipofectin method (GIBCO BRL).

Biochemical Methods

After ligation of integrins (Mainiero et al., 1995), cells were extracted and subjected to immunoprecipitation followed by immunoblotting or kinase assay. To immunoprecipitate Shc, integrins, caveolin, and FAK, cells were extracted in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100. To immunoprecipitate Erk2, cells were extracted with 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA. Immunoprecipitation, immunoblotting, and GST fusion protein binding experiments were performed as previously described (Mainiero et al., 1995). Erk2 immune-complex kinase assays were performed in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ containing 5 μCi of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Biomedicals, Incorporation) and 2.5 μg of MHC-binding protein (MBP). Ras–GTP loading assays were performed as described (Gale et al., 1993). To measure SRE-dependent transcription, HUVECs were transiently transfected with Fos-SRE-Luc. After growth factor starvation, the cells were detached, plated on dishes coated with 10 μg/ml poly-L-lysine, fibronectin, vitronectin, laminin

1, or laminin 4, and then subjected to luciferase assay. At this coating concentration, all extracellular matrix proteins promoted similar levels of adhesion and spreading in HUVECs.

Measurement of Cell Cycle Progression and Apoptosis

To monitor progression through G1 and entry into S phase, HUVECs were synchronized in G0 by growth factor starvation, detached, and plated at low density on microtiter plates or glass coverslips coated with poly-L-lysine or extracellular matrix molecules. After 24 hr of incubation in defined medium (M199 supplemented with 25 ng/ml bFGF, 1 μg/ml heparin, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 0.625 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 μg/ml linoleic acid) containing 10 μM BrdU, cells were stained with anti-BrdU MAb and AP-conjugated anti-mouse IgGs (Boehringer).

To test the effect of dominant-negative and wild-type Shc on cell cycle progression, HUVECs were transiently transfected with vectors encoding FLAG-tagged dominant-negative Shc, FLAG-tagged wild-type Shc, or the control protein HA-β-galactosidase. After incubation in complete medium for 8 hr, the cells were starved for 24 hr and then incubated in defined medium containing 10 μM BrdU for 24 hr. Entry into S phase of transfected cells was monitored by double immunofluorescent staining with anti-FLAG MAb M2 or anti-HA MAb 12CA5 followed by Texas red–conjugated anti-mouse IgGs and FITC-conjugated anti-BrdU antibodies.

To measure apoptosis, G0-synchronized HUVECs were plated on wells or coverslips coated with poly-L-lysine or extracellular matrix proteins and incubated in defined medium for the indicated times. Attached and unattached cells were combined and stained in suspension with Hoechst dye.

Acknowledgments

Correspondence should be addressed to F. G. G. We thank D. Bar-Sagi, D. Cheresh, C. Damsky, R. Juliano, S. Kennel, S. LaFlamme, S. Retta, E. Scolnik, J. Schlessinger, G. Tarone, and K. Yamada for reagents, B. Gumbiner and E. Scolnick for reviewing the manuscript, M. Palmieri for the analysis of cells expressing mutant β_1 subunits, F. David and A. Kern for FACS analysis, and A. Pepe and M. Epstein for technical assistance. This work was supported by Department of Army Medical Defense grant 17-94-J4306 (F. G. G.) and National Institutes of Health (NIH) grants CA-58976 (F. G. G.), GM-44585 (E. E. M.), and CA–16087 (Cancer Center). K. K. W. was supported by NIH training grant CA-09161. F. G. G. is an Established Investigator of the American Heart Association (AHA). E. E. M. is an Established Scientist of the New York City Affiliate of the AHA.

Received June 26, 1996; revised September 27, 1996.

References

Bauer, J.S., Varner, J., Schreiner, C., Kornberg, L., Nicholas, R., and Juliano, R.L. (1993). Functional role of the cytoplasmic domain of the integrin α_5 subunit. J. Cell Biol. *122*, 209–221.

Briesewitz, R., Kern, A., and Marcantonio, E.E. (1993). Ligand-dependent and -independent integrin focal contact localization: the role of the α chain cytoplasmic domain. Mol. Biol. Cell *4*, 593–604.

Brooks, P.C., Montgomery, A.M.P., Rosenfeld, M., Reisfeld, R.A., Hu, T., Klier, G., and Cheresh, D.A. (1994). Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell *79*, 1157–1164.

Chen, H.-C., and Guan, J.-L. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. USA. *91*, 10148–10152.

Chen, Y., Grall, D., Salcini, A.E., Pelicci, P.G., Pouyssegur, J., and Van Obberghen-Schilling, E. (1996). Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein–coupled thrombin receptor. EMBO J. *15*, 1037–1044.

Cheresh, D.A. (1987). Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and Von Willebrand factor. Proc. Natl. Acad. Sci. USA. *84*, 6471–6475.

Clark, E.A., and Brugge, J.S. (1995). Integrins and signal transduction pathways: the road taken. Science *268*, 233–239.

Conforti, G., Zanetti, A., Colella, S., Abbadini, M., Marchisio, P.C., Pytela, R., Giancotti, F.G., Tarone, G., Languino, L.R., and Dejana, E. (1989). Interaction of fibronectin with cultured human endothelial cells: characterization of the specific receptor. Blood *73*, 1576–1585.

Corley Mastick, C., Brady, M.J., and Saltiel, A.R. (1995). Insulin stimulates the tyrosine phosphorylation of caveolin. J. Cell Biol. *129*, 1523–1531.

Cowley, S., Patterson, H., Kemp, P., and Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell *77*, 841–852.

Fang, F., Orend, G., Watanabe, N., Hunter, T., and Ruoslahti, E. (1996). Dependence of cyclin E–CDK 2 kinase activity on cell anchorage. Science *271*, 499–502.

Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J., and Bar-Sagi, D. (1993). Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature *363*, 88–92.

Giancotti, F.G., and Mainiero, F. (1994). Integrin-mediated adhesion and signaling in tumorigenesis. Biochem. Biophys. Acta *1198*, 47–64.

Giancotti, F.G., and Ruoslahti, E. (1990). Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell *60*, 849–859.

Giancotti, F.G., Spinardi, L., Mainiero, F., and Sanders, R. (1994). Expression of heterologous integrin genes in cultured eukaryotic cells. Methods Enzymol. *245*, 297–316.

Glenney, J.R., and Soppet, D. (1992). Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus–transformed fibroblasts. Proc. Natl. Acad. Sci. USA. *89*, 10517–10521.

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309–324.

Huhtala, P., Humphries, M.J., McCarthy, J.B., Tremble, P.M., Werb, Z., and Damsky, C.H. (1995). Cooperative signaling by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins regulate metalloproteinase gene expression in fibroblasts adhering to fibronectin. J. Cell Biol. *129*, 867–879.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Hynes, R.O. (1996). Targetted mutations in cell adhesion genes: what have we learned from them? Dev. Biol., in press.

Ilić, D., Fruta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539–544.

Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E.E. (1994). The role of the I domain in ligand binding of the human integrin $\alpha_1\beta_1$. J. Biol. Chem. *269*, 22811–22816.

Kubota, W., Kleinman, H.K., Martin, G.R., and Lawley, T.J. (1988). Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol. *107*, 1589–1598.

LaFlamme, S.E., Akiyama, S.K., and Yamada, K.M. (1992). Regulation of fibronectin receptor distribution. J. Cell Biol. *117*, 437–447.

Languino, L.R., Gehlsen, K.R., Wayner, E., Carter, W.G., Engvall, E., and Ruoslahti, E. (1989). Endothelial cells use $\alpha_2\beta_1$ integrins as a laminin receptor. J. Cell Biol. *109*, 2455–2462.

Lin, C.Q., and Bissell, M.J. (1993). Multi-faceted regulation of cell differentiation via extracellular matrix. FASEB J. *7*, 737–743.

Lisanti, M.P., Scherer, P.E., Tang, Z., and Sargiacomo, M. (1994). Caveolae, caveolin, and caveolin-rich membrane domains: a signaling hypothesis. Trends Cell Biol. *4*, 231–235.

Mainiero, F., Pepe, A., Wary, K.K., Spinardi, L., Mohammadi, M., Schlessinger, J., and Giancotti, F.G. (1995). Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. EMBO J. *14*, 4470–4481.

Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W.H., Jaye, M., and Schlessinger, J. (1996). Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol. Cell. Biol. *16*, 977–989.

Pawson, T. (1995). Protein modules and signaling networks. Nature *373*, 573–580.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P.G. (1992) A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. Cell *70*, 93–104.

Pullan, S., Wilson, J., Metcalfe, A., Edwards, G.M., Goberdhan, N., Tilly, J., Hickman, J.A., Dive, C., and Streuli, C.H. (1996). Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. J. Cell Sci. *109*, 631–642.

Richardson, A., and Parsons, T. (1996). A mechanism for regulation of the adhesion-associated protein tyrosine kinase $pp125^{FAK}$. Nature *380*, 538–540.

Ruoslahti, E. (1991). Integrins. J. Clin. Invest. *87*, 1–5.

Ruoslahti, E., and Reed, J.C. (1994). Anchorage dependence, integrins, and apoptosis. Cell *77*, 477–478.

Salcini, A.E., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T., and Pelicci, P.G. (1994). Formation of Shc–Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. Oncogene *9*, 2827–2836.

Sastry, S.K., Lakonishok, M., Thomas, D.A., Muschler, J., and Horwitz, A.F. (1996). Integrin α subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. J. Cell Biol. *133*, 169–184.

Schaller, M.D., and Parsons, J.T. (1994). Focal adhesion kinase and associated proteins. Curr. Opin. Cell Biol. *6*, 705–710.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature *372*, 786–791.

Skapek, S., Rhee, J., Spicer, D.B., and Lassar, A.B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1–dependent kinase. Science *267*, 1022–1024.

Spinardi, L., Einheber, S., Cullen., T., Milner, T.A., and Giancotti, F. G. (1995). A recombinant tail-less integrin $\alpha_6\beta_4$ subunit disrupts hemidesmosomes, but does not suppress $\alpha_6\beta_4$-mediated cell adhesion to laminins. J. Cell Biol. *129*, 473–487.

Treisman, R. (1995). Journey to the surface of the cell: Fos regulation and SRE. EMBO J. *14*, 4905–4913.

von der Mark, K., and Ocalan, M. (1989). Antagonistic effects of laminin and fibronectin on the expression of the myogenic phenotype. Differentiation *40*, 150–157.

Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996). Induction of $p130^{CAS}$ signaling complex formation upon integrin-mediated cell adhesion: a role for *src* family kinases. Mol. Cell. Biol. *16*, 2606–2613.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., and Greenberg, M.E. (1995). Opposing effects of Erk and Jnk–p38 MAP kinases on apoptosis. Science *270*, 1326–1331.

Zhang, Z., Vuori, K., Reed, J.C., and Ruoslahti, E. (1995). The $\alpha_5\beta_1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc. Natl. Acad. Sci. USA. *92*, 6161–6165.

Zhu, X., and Assoian, R.K. (1995). Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol. Biol. Cell *6*, 273–282.

Zhu, X., Ohtsubo, M., Bohmer, R., Roberts, J.M., and Assoian, R.K. (1996). Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E–cdk2, and phosphorylation of the retinoblastoma protein. J. Cell Biol. *133*, 391–403.

The EMBO Journal Vol.17 No.14 pp.3940–3951, 1998

Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin β4 cytoplasmic domain

Chiara Murgia[1,2], Pamela Blaikie[1], Nancy Kim[1], Michael Dans[1], Howard T.Petrie[3,4] and Filippo G.Giancotti[1,4,5]

[1]Cellular Biochemistry and Biophysics Program and [3]Immunology Program, Memorial Sloan-Kettering Cancer Center and [4]Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, NY 10021, USA

[2]Present address: Istituto Nazionale della Nutrizione, via Ardeatina, 546, Roma, Italy

[5]Corresponding author
e-mail: F-Giancotti@ski.mskcc.org

The cytoplasmic domain of the integrin β4 subunit mediates both association with the hemidesmosomal cytoskeleton and recruitment of the signaling adaptor protein Shc. To examine the significance of these interactions during development, we have generated mice carrying a targeted deletion of the β4 cytoplasmic domain. Analysis of homozygous mutant mice indicates that the tail-less α6β4 binds efficiently to laminin 5, but is unable to integrate with the cytoskeleton. Accordingly, these mice display extensive epidermal detachment at birth and die immmediately thereafter from a syndrome resembling the human disease junctional epidermolysis bullosa with pyloric atresia (PA-JEB). In addition, we find a significant proliferative defect. Specifically, the number of precursor cells in the intestinal epithelium, which remains adherent to the basement membrane, and in intact areas of the skin is reduced, and post-mitotic enterocytes display increased levels of the cyclin-dependent kinase inhibitor $p27^{Kip}$. These findings indicate that the interactions mediated by the β4 tail are crucial for stable adhesion of stratified epithelia to the basement membrane and for proper cell-cycle control in the proliferative compartments of both stratified and simple epithelia.

Keywords: blistering skin disease/cell cycle/gene targeting/integrin/signaling

Introduction

Basement membranes are thin, continuous sheets of specialized extracellular matrix (ECM), which support epithelial and other cells and separate them from the underlying interstitial connective tissue (Yurchenco and O'Rear, 1994; Timpl and Brown, 1996). In addition to promoting cell adhesion and cytoskeletal organization, basement membranes influence the proliferation and differentiation of cells. The effects of basement membranes on cellular behavior are likely to be mediated by integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler, 1990). Upon binding to ECM ligand, integrins cluster on the plasma membrane and interact with the cytoskeleton, thereby promoting the assembly of adhesive junctions, such as focal adhesions and hemidesmosomes (Borradori and Sonnenberg, 1996; Burridge and Chrzanowska-Wonidka, 1996). In addition, integrins are coupled to intracellular signaling pathways potentially able to regulate the cell cycle (Giancotti, 1997). In particular, a class of integrins, which include the α6β4 laminin receptor, the α1β1 collagen IV/laminin receptor, the α5β1 fibronectin receptor and the broad specificity receptor αvβ3, are linked to the Ras-ERK signaling pathway and control the cell cycle by the signaling adaptor protein Shc (Mainiero *et al.*, 1995, 1997; Wary *et al.*, 1996).

The integrin α6β4 is a receptor for various isoforms of the basement membrane component laminin and binds with the highest apparent affinity to laminin 5 (Giancotti, 1996). *In vivo*, α6β4 is expressed in a number of tissues and cell types that are in contact with a basement membrane, including both simple and stratified epithelia (Kajiji *et al.*, 1989), Schwann cells (Sonnenberg *et al.*, 1990; Einheber *et al.*, 1993), and a subset of endothelial cells (Kennel *et al.*, 1992; Klein *et al.*, 1993) and thymocytes (Wadsworth *et al.*, 1992). In contrast to all the other known α and β subunit cytoplasmic domains, which are relatively short, the intracellular portion of the β4 subunit measures over 1000 amino acids in length and contains, in its C-terminal half, two pairs of type III fibronectin-like repeats separated by a 142 amino acid connecting segment (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). While β1 and αv subunit-containing integrins interact with the actin cytoskeleton and localize to focal adhesions, α6β4 is found concentrated at hemidesmosomes both in cultured cells and *in vivo* (Carter *et al.*, 1990; Stepp *et al.*, 1990), suggesting that the β4 tail specifies association with the hemidesmosomal cytoskeleton.

Hemidesmosomes are complex adhesive junctions that link the basement membrane to the intracellular keratin cytoskeleton and are found exclusively in the basal cell layer of stratified and transitional epithelia (Borradori and Sonnenberg, 1996). Gene transfer studies in cultured cells have provided evidence that the unique cytoplasmic domain of β4, and specifically a region comprising the first pair of type-III fibronectin-like modules and the connecting segment, is required for association of α6β4 with the hemidesmosomal cytoskeleton (Spinardi *et al.*, 1993). Subsequent studies have indicated that ligation of α6β4 activates an integrin-associated kinase and causes phosphorylation of the β4 cytoplasmic domain at multiple tyrosine residues (Mainiero *et al.*, 1995). Phosphorylation of a tyrosine activation motif (TAM) located in the connecting segment is likely to be required for association with the hemidesmosomal cytoskeleton, because mutations at either one of the two tyrosine residues in the TAM abolish the incorporation of α6β4 in hemidesmosomes

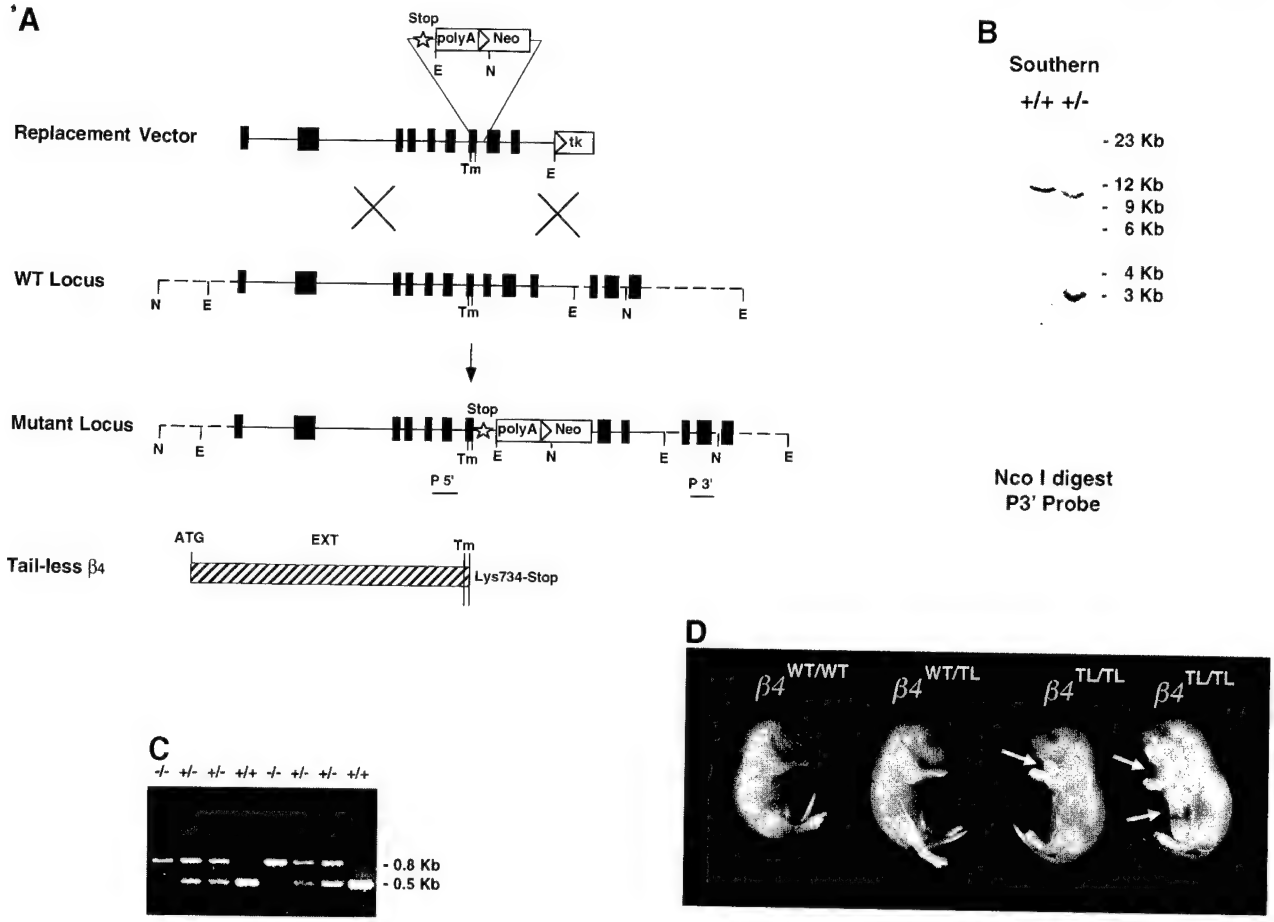


Fig. 1. Targeted deletion of the β4 cytoplasmic domain. (**A**) Strategy used to introduce a stop codon in the β4 gene. The structures of replacement vector, wild-type (WT) locus, mutant locus and tail-less β4 protein are shown. The star indicates the Stop codon, which is followed by an SV40 polyadenylation signal and a neomycin resistance expression cassette. Restriction sites are indicated: E, *Eco*RI; N, *Nco*I. The sequences encoding the transmembrane segment of β4 are indicated (Tm). P5′ and P3′ are DNA probes used for Southern hybridization. (**B**) Southern analysis on genomic DNA from wild-type (+/+) and heterozygous mutant (+/–) ES clones. (**C**) PCR analysis on the intercrosses between heterozygous mutant mice: wild-type (+/+), heterozygous mutant (+/–) and homozygous mutant (–/–) mice. The 0.8 kb band originates from the mutant allele, the 0.5 kb band from the wild-type allele. (**D**) A wild-type ($\beta4^{WT/WT}$), a heterozygous mutant ($\beta4^{WT/TL}$) and two homozygous mutant mice ($\beta4^{TL/TL}$) with different degrees of epidermal blistering (arrows) are shown.

(Mainiero *et al.*, 1995). The ability of a truncated β4 subunit to exert a dominant-negative effect on hemidesmosome assembly without inhibiting initial adhesion to laminin 5 (Spinardi *et al.*, 1995) and the absence of hemidesmosomes in the skin of α6 and β4 knock-out mice (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996) have indicated that α6β4 plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system. Although it is widely assumed that integrin interaction with the cytoskeleton at adhesive junctions such as hemidesmosomes is necessary to consolidate cell adhesion (Alberts *et al.*, 1994), this hypothesis has not yet been tested directly *in vitro* or *in vivo*. For example, although mice carrying null mutations at the β4 or α6 locus die a few hours after birth from a severe form of epidermal blistering (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996), it is not clear whether their cell adhesion defect is caused by the absence of α6β4-mediated binding to laminin 5, the lack of integrin association with the cytoskeleton, or both.

Several observations suggest that ligation of α6β4 may also promote cell proliferation. In the skin and other stratified squamous epithelia, the expression of α6β4 is restricted to the actively proliferating basal keratinocytes (Kajiji *et al.*, 1989). When these cells detach from the laminin-rich basement membrane to migrate to the upper epidermal layers, they exit from the cell cycle and begin to differentiate (Hall and Watt, 1989). Similarly, depriving cultured keratinocytes of anchorage to their ECM, which is rich in laminin 5 (Carter *et al.*, 1991; Rousselle *et al.*, 1991), results in withdrawal from the cell cycle and differentiation (Green, 1977). Finally, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of α6β4 (Kimmel and Carey, 1986; Wolf *et al.*, 1990), and overexpression of the integrin augments the invasive potential of breast carcinoma cells (Shaw *et al.*, 1997). These results suggest that α6β4 can facilitate both invasion and growth in tumor cells.

In accordance with these observations, recent biochemical studies have indicated that α6β4 activates signaling pathways able to influence cell proliferation. In particular, ligation of α6β4 results in recruitment of the adaptor protein Shc. Upon association with α6β4, Shc is phosphorylated on tyrosine and thereby combines with the Grb2/mSOS complex (Mainiero *et al.*, 1995). This process

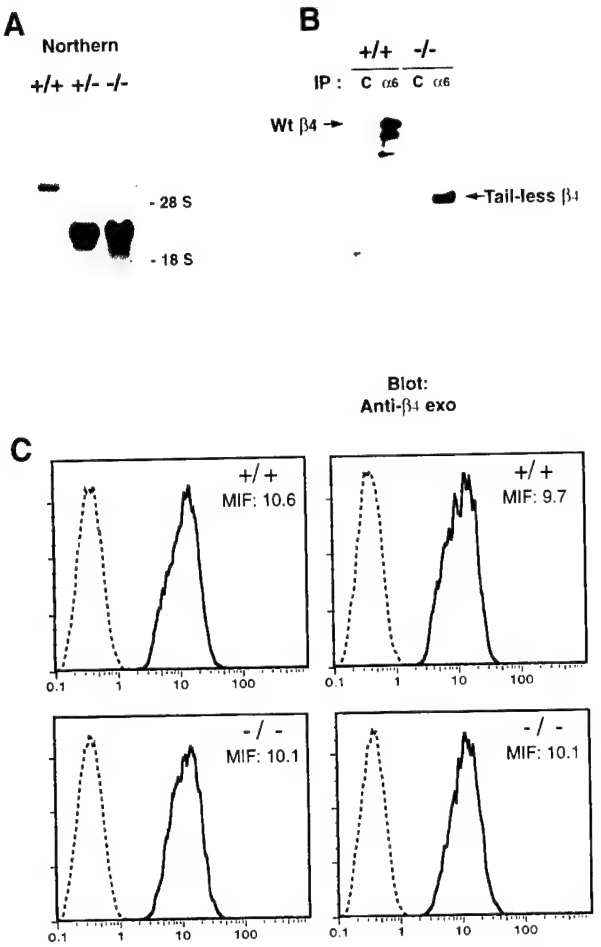


Fig. 2. Expression analysis. (**A**) An aliquot (10 μg) of total RNA extracted from the skin of wild-type (+/+), heterozygous (+/–) and homozygous mutant (–/–) E18.5 embryos was probed with a 0.5 kb mouse cDNA fragment complementary to the extracellular portion of β4 mRNA. (**B**) Equal amounts of total epidermal proteins extracted from the skin of wild-type and homozygous mutant E18.5 embryos were immunoprecipitated with anti-α6 mAb GoH3 (α6) or control anti-MHC mAb W6.32 (C) and probed by immunoblotting with a rabbit antiserum to the ectodomain of β4. Arrows point to wild-type and tail-less β4. The two lower molecular weight forms of wild-type β4 correspond to the proteolytic processing products A and B described previously (Giancotti *et al.*, 1992). (**C**) $CD25^+$ (solid lines) and $CD25^-$ (dashed lines) thymocytes from wild-type and homozygous mutant E16.5 embryos were subjected to FACS analysis with mAb 346-11A, which binds to the extracellular domain of mouse β4. Mean intensities of fluorescence (MIF) for $CD25^+$ cells are indicated.

results in the activation of both the Ras-ERK and the Rac-JNK mitogen-activated protein (MAP) kinase pathways (Mainiero *et al.*, 1997). The α6β4 integrin has also been shown to activate phosphatidylinositol 3-OH kinase (PI-3K) (Shaw *et al.*, 1997). Since inhibition of PI-3K blocks the activation of JNK by α6β4, it is likely that this lipid kinase couples α6β4-mediated activation of Ras to the Rac-JNK pathway (Mainiero *et al.*, 1997). In cultured keratinocytes, adhesion to laminin 5 mediated by α6β4 induces transcription from the Fos serum response element (SRE) and promotes progression through the G_1 phase of the cell cycle in the presence of epidermal growth factor (EGF). In contrast, adhesion mediated by α2β1, which is not linked to Shc signaling, results in exit from the cell cycle even in the presence of otherwise mitogenic concentrations of EGF (Mainiero *et al.*, 1997). These findings suggest that the signals from α6β4 and the EGF receptor converge on the Ras-ERK pathway, ultimately resulting in transcription of immediate-early genes and progression through G_1. Although it is clear that α6β4 can promote cell proliferation under defined conditions *in vitro*, it remains to be established whether α6β4 signaling influences cell proliferation *in vivo*.

In order to examine the biological significance of the intracellular functions of α6β4 *in vivo*, we have generated mice carrying a targeted deletion of the β4 cytoplasmic domain. Analysis of these mice indicates that the β4 cytoplasmic domain is necessary for stable adhesion of stratified epithelia to the basement membrane and for proper cell cycle control in both simple and stratified epithelia.

Results and discussion

Targeted deletion of the β4 cytoplasmic domain

Homologous recombination in embryonic stem (ES) cells was used to introduce a stop codon in the mouse β4 gene immediately after the sequences encoding the transmembrane domain. As illustrated in Figure 1A, the replacement vector consisted of a fragment of ~7 kb of the mouse β4 gene interrupted immediately after the exon encoding the transmembrane segment by an insertion cassette containing a stop codon followed by an SV40 polyadenylation site and a neomycin resistance gene. Homologous recombination at the wild-type locus was expected to generate a mutant locus encoding a β4 subunit truncated after Lys734, the halt-transfer stop signal. Gene transfer experiments in cultured cells have indicated that this tail-less β4 subunit combines efficiently with α6 and is exported regularly to the cell surface, but is unable to associate with Shc (Mainiero *et al.*, 1997) or the hemidesmosomal cytoskeleton (data not shown). Southern blot analysis indicated successful incorporation of the desired mutation in ~10% of the doubly selected ES cell clones (Figure 1B and data not shown). PCR analysis on the intercrosses between heterozygous mice carrying the targeted deletion revealed that the mutation was transmitted with the expected Mendelian frequency (Figure 1C). While heterozygous mice appeared completely normal, all homozygous mutant animals presented with extensive blistering of the skin, particularly in the abdomen, the ventral portion of the torso, the paws and the tip of the tail (Figure 1D). They were unable to feed, and died within a few hours after birth, presumably from dehydration. To minimize damage to the skin, all subsequent analyses were conducted on mice delivered by Cesarean section by embryonic day (E) 18.5.

Expression analysis

To examine if the tail-less β4 subunit encoded by the mutant allele was expressed correctly, we subjected the skin of wild-type and mutant mice to Northern blotting and immunoprecipitation analysis. As shown in Figure 2A, the recombinant truncated mRNA had the expected size, but was significantly more abundant than the wild-type mRNA, presumably as a result of increased stability and/or transcription. If the biosynthesis of β4 exceeds that of α6, the fraction of unpaired β4 is degraded rapidly in

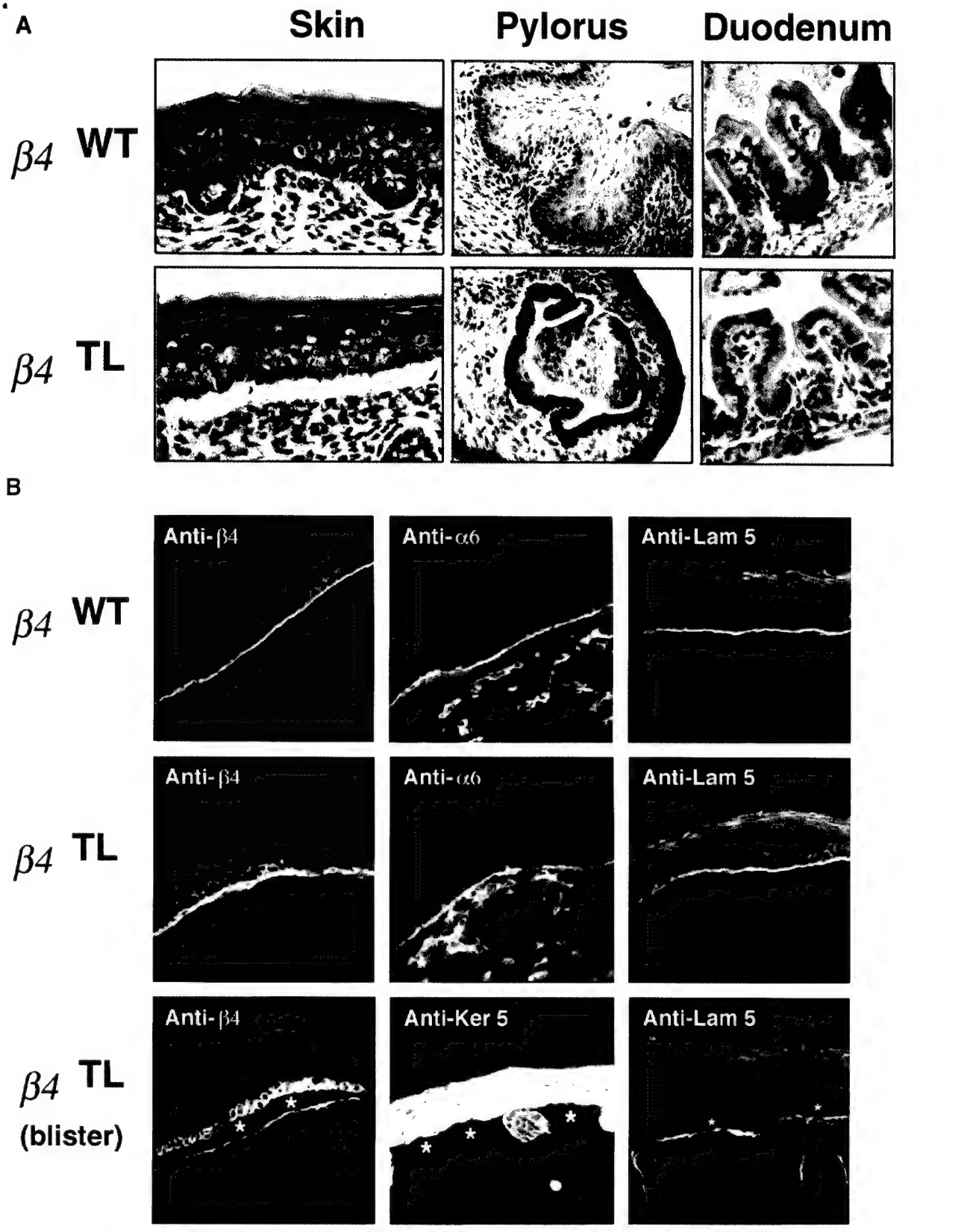


Fig. 3. The stratified epithelium of mutant mice detaches from the basement membrane leaving a fraction of tail-less α6β4 behind.
(**A**) Hematoxilyn–eosin staining of skin and pyloric junction and periodic acid Shiff (PAS)–hematoxylin staining of proximal duodenum from wild-type ($\beta4^{WT}$) and homozygous mutant ($\beta4^{TL}$) E18.5 embryos. PAS-positive intestinal goblet cells are stained in red. (**B**) Sections from the skin of $\beta4^{WT}$ embryos and attached and unattached areas of the skin of $\beta4^{TL}$ embryos at E18.5 were subjected to immunofluorescent staining with mAb 346-11A which binds to the extracellular domain of mouse β4 (anti-β4), mAb GoH3 (anti-α6), affinity-purified rabbit antibodies to mouse keratin 5, which at this stage is expressed in both basal and suprabasal layers of epidermis (anti-Ker 5), and a rabbit antiserum to human laminin 5 (anti-Lam 5). The stars mark the areas of separation of the epidermis from the basement membrane.

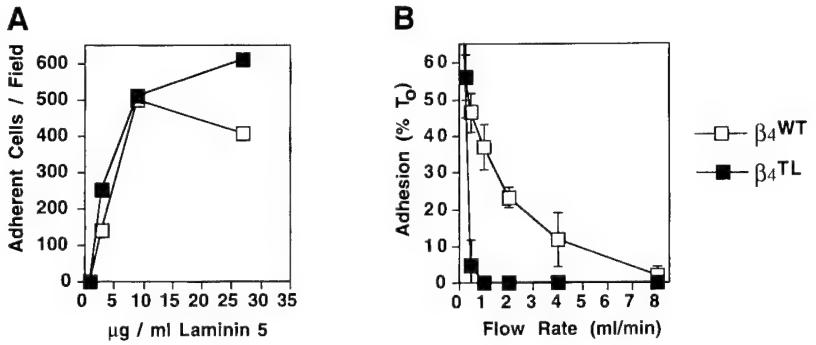


Fig. 4. Tail-less α6β4 does not mediate stable adhesion to laminin 5 *in vitro*. (**A**) Static adhesion assay. Primary keratinocytes from $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were incubated in the presence of the inhibitory anti-mouse β1 mAb HMβ1-1 on microtiter wells coated with the indicated concentrations of purified laminin 5 for 30 min. (**B**) Laminar flow detachment assay. Cells were drawn into glass capillaries coated with 10 µg/ml purified laminin 5 and allowed to adhere under static conditions for 30 min in the presence of the inhibitory anti-β1 mAb HMβ1-1. The cells were then subjected to increasing flow rates. The number of cells remaining adherent after each step is expressed as a percentage of cells adhering at time 0 (T_0).

the endoplasmic reticulum (ER) (Giancotti *et al.*, 1992). In accordance with this finding, immunoprecipitation experiments indicated that the amount of tail-less β4 associated with α6 in homozygous mutant mice was comparable with that of wild-type β4 paired with α6 in wild-type mice (Figure 2B). To verify that the levels of tail-less α6β4 at the cell surface were comparable with those of wild-type integrin, we performed fluorescence-activated cell sorting (FACS) analysis on $CD25^+$ thymocytes derived from homozygous mutant and wild-type mice. These cells express homogeneous levels of α6β4 at their surface (Wadsworth *et al.*, 1992) and can be analyzed easily without subculturing. The results demonstrated that the level of expression of tail-less α6β4 at the cell surface was similar to that of wild-type integrin (Figure 2C). Similar results were obtained with primary keratinocytes (not shown). We concluded that the tail-less integrin encoded by the mutant allele was expressed correctly at the cell surface.

Characterization of the cell adhesion defect

Histological analysis of the skin of mutant mice showed wide areas of separation of the epidermis from the dermis (Figure 3A). In several instances, the epidermis remained connected to the dermis by short filaments emanating from the basal keratinocytes. Cross-sections at the pyloric junction revealed that the stratified epithelium of the esophagus had detached from the underlying connective tissue and degenerated (Figure 3A). Similar signs of degeneration were also observed in cross-sections of the largest (and presumably oldest) epidermal blisters. No signs of apoptosis in either affected or unaffected areas of the skin were revealed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis (data not shown), and electron microscopy indicated that cell death in the oldest blisters was due to necrosis, presumably from the interruption of nutrient exchange with the dermal interstitium (Figure 6, bottom left panel). These results are consistent with the observation that primary keratinocytes denied anchorage to the ECM exit from the cell cycle and begin to differentiate rather than undergoing apoptosis (Gandarillas *et al.*, 1997). The histological abnormalities of tail-less β4 mice resembled those observed in the human disease junctional epidermolysis bullosa with pyloric atresia (PA-JEB), which is caused by mutations in the α6 or β4 gene (Vidal *et al.*, 1995; Ruzzi *et al.*, 1997), as well as in α6 and β4 knock-out mice (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996). In contrast to the epidermis, the intestinal epithelium of homozygous mutant mice did not detach from the underlying mesenchyme (Figure 3A), suggesting that the cytoplasmic domain of β4 is essential for stable adhesion of stratified, but not simple, epithelia. No signs of apoptosis were detected in either wild-type or mutant intestinal epithelium at E18.5 (data not shown). Thus, signaling by the cytoplasmic domain of β4 may contribute to, but does not appear to be required for, epithelial survival.

We next examined the mechanism of epidermal detachment in mutant mice. Immunofluorescent staining of unaffected areas of the skin indicated that the tail-less α6β4 was concentrated normally at the basement membrane junction. This suggests that the tail-less integrin binds efficiently to laminin 5, and this binding is sufficient for recruitment to the basal surface in the absence of cytoskeletal interactions (Figure 3B). The immunostaining for laminin 5 (as well as collagen IV and laminin 1, data not shown) in mutant mice was linear along the basement membrane and had the same intensity as that observed in normal mice. The pattern of expression of other epidermal integrins, including α1β1, α2β1 and the basement membrane assembly receptor α3β1 (DiPersio *et al.*, 1997), was also unchanged in the skin of mutant mice (data not shown). Finally, the ultrastructure of basement membrane in the skin of mutant mice appeared normal at the electron microscopic level (Figure 6, top right). These observations suggest that the mutation in β4 does not affect the expression of other integrins or the assembly of the basement membrane.

Immunofluorescent analysis of detached areas of the skin of mutant mice indicated that the anti-laminin 5 staining demarcated the floor of the lesions, indicating that the skin had split above the basement membrane. However, a significant fraction of tail-less α6β4 remained attached to the basement membrane, while keratin 5 was confined entirely to the epidermis above the roof of the blister (Figure 3B). In accordance with this finding, electron microscopic analysis indicated that remnants of basal cell projections remained attached to the basement

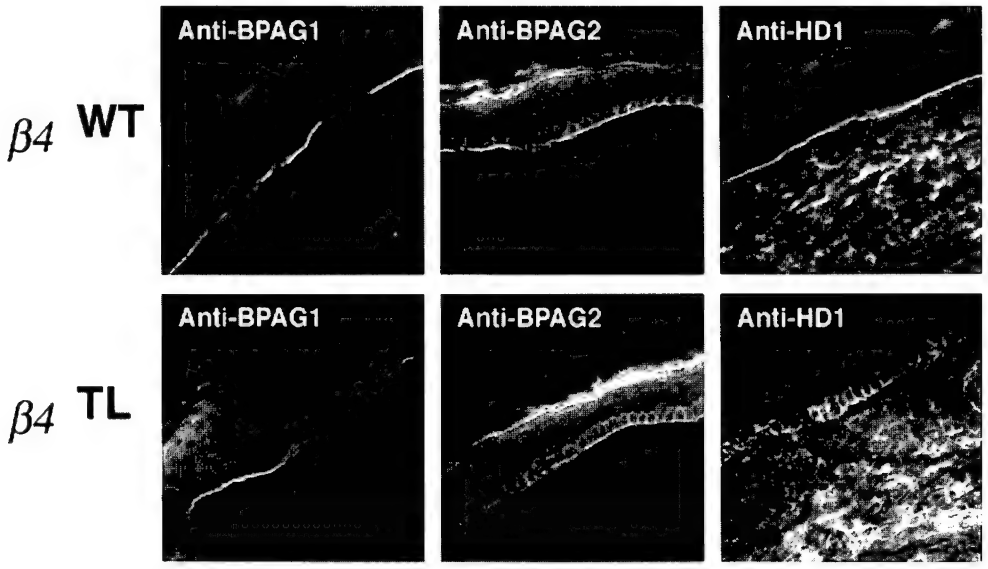


Fig. 5. Abnormal recruitment of HD1/plectin to the basement membrane zone in the epidermis of tail-less β4 mice. Skin sections from $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were subjected to immunofluorescent staining with human mAb 5E (anti-BPAG1), rabbit antiserum to BPAG2 (anti-BPAG2) and mAb 121 (anti-HD1).

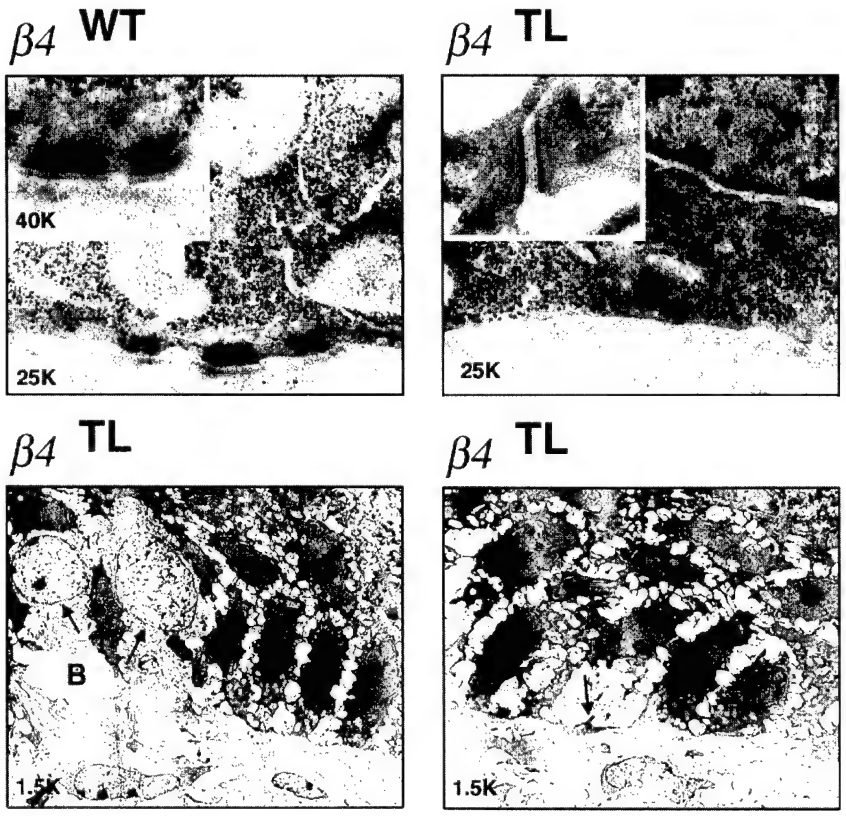


Fig. 6. Absence of hemidesmosomes in the epidermis of tail-less β4 mice. The epidermal–dermal junction of $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos was examined by transmission electron microscopy. Note the presence of hemidesmosomes in the skin of $\beta4^{WT}$ embryos (top left) and their absence in the skin of $\beta4^{TL}$ embryos (top right). Desmosomes are intact in $\beta4^{WT}$ embryos (top right, insert). In the skin of $\beta4^{TL}$ embryos, basal keratinocytes remaining in contact with the basement membrane do not show signs of apoptosis, while those which have detached from the basement membrane display cytoplasmic and nuclear features of necrosis. Arrows point to the nuclei of two necrotic keratinocytes above a small blister (B) (bottom left). Isolated detaching keratinocytes often left fragments of their basal portion attached to the basement membrane (bottom right, arrow). The separation between epidermal cells in the lower panels is an artifact of fixation. Magnifications are indicated in the lower left corner of each panel.

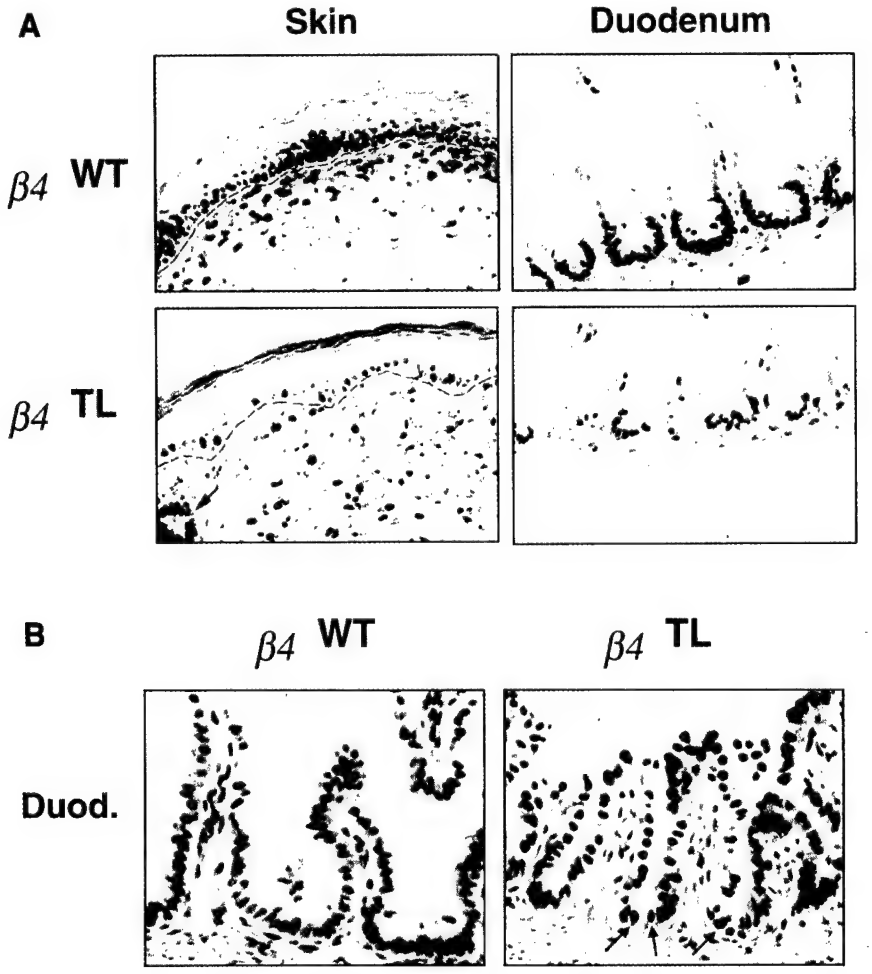


Fig. 7. Proliferation defects in the epidermis and intestinal epithelium of tail-less β4 mice. (**A**) Sections from the skin and duodenum of $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were subjected to immunoperoxidase staining with the anti-Ki-67 mAb MM1 and counterstained with hematoxylin. (**B**) Duodenal sections from $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were subjected to immunoperoxidase staining with affinity-purified anti-p27 antibodies and counterstained with hematoxylin. Arrows point to p27-positive cells in the intervillar region.

membrane (Figure 6, bottom right panel). The observation that a significant fraction of tail-less α6β4 appears to be pulled out of the membrane of basal keratinocytes when these cells detached from the basement membrane is consistent with the finding that the tail-less integrin binds efficiently to laminin 5, but cannot integrate with the cytoskeleton.

To investigate further the adhesive function of tail-less α6β4, we performed adhesion assays on primary keratinocytes derived from wild-type and homozygous mutant mice. The cells were plated on microtiter wells coated with different concentrations of laminin 5 in the presence of a monoclonal antibody that blocks the adhesive function of all β1 integrins, including α3β1, the only other known laminin 5 receptor in keratinocytes. As shown in Figure 4A, the keratinocytes expressing tail-less α6β4 adhered to laminin 5 as well as cells expressing the wild-type integrin in an adhesion assay. However, the results of a laminar flow detachment assay revealed that the adhesive strength mediated by tail-less α6β4 was significantly lower than that of the wild-type integrin (Figure 4B). These results indicate that while the tail-less integrin binds efficiently to laminin 5, it is unable to mediate stable adhesion.

To examine the role of the β4 tail in hemidesmosome assembly, non-lesional areas of the skin of mutant mice were subjected to immunofluorescence and electron microscopic analysis. Immunofluorescent staining indicated that the cytoskeletal component HD1/plectin, which interacts with the β4 cytoplasmic domain (Niessen *et al.*, 1997; Rezniczek *et al.*, 1998), was distributed diffusely in the cytoplasm of basal keratinocytes instead of being concentrated at the basement membrane junction (Figure 5). The putative adhesion receptor bullous pemphigoid antigen 2 (BPAG2), which is thought to be recruited to hemidesmosomes in response to a signal generated by the β4 cytoplasmic domain (Borradori *et al.*, 1997), was partially polarized at the basal surface of keratinocytes in both wild-type and mutant mice, suggesting that the ability of BPAG2 to bind to an extracellular ligand may be sufficient for targeting at the basement membrane junction (Figure 5). The hemidesmosomal plaque component BPAG1, which is involved in interaction with the keratin cytoskeleton (Guo *et al.*, 1995), was concentrated at the basement membrane junction despite the absence of the β4 tail (Figure 5), perhaps because it can interact at least indirectly with BPAG 2. These observations indicate that the cytoplasmic domain of β4 is necessary for the recruitment of HD1/plectin, but not BPAG2 and BPAG1, to the basement membrane zone.

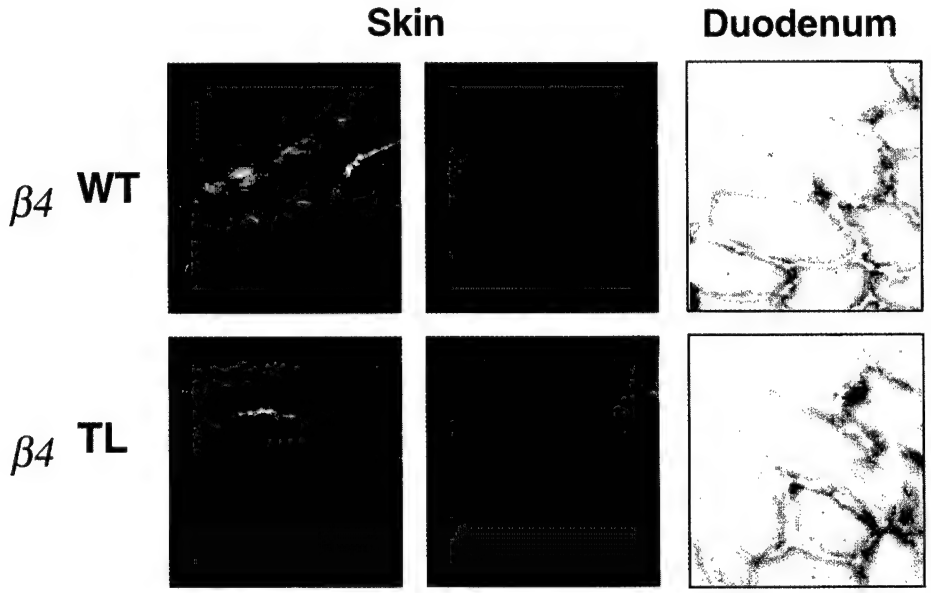


Fig. 8. Normal expression of differentiation markers in the epidermis and intestinal epithelium of tail-less β4 mice. Skin sections from $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were subjected to either double immunostaining with antibodies to loricrin (red) and laminin β2 subunit (green) or single immunostaining with antibodies to filaggrin. Duodenal sections from $\beta4^{WT}$ and $\beta4^{TL}$ E18.5 embryos were assayed for lactase activity (Stoward and Everson Pearse, 1991) (blue).

Finally, electron microscopic analysis revealed that the epidermis of mutant mice completely lacked hemidesmosomes (Figure 6, top right). The desmosomes were instead intact (Figure 6, top right, insert). Taken together, these results demonstrate that the β4 cytoplasmic domain is necessary for nucleation of hemidesmosomes and indicate that the adhesive defect of mutant mice is caused by the inability of tail-less α6β4 to mediate this function.

Analysis of the cell signaling defect

In the next series of experiments, we analyzed if α6β4 signaling regulated cell proliferation in stratified and simple epithelia. In the adult epidermis, the expression of α6β4 is restricted to the basal layer, which contains cells with proliferative capacity (Giancotti, 1996). To examine the role of α6β4 signaling in epidermal proliferation, skin sections from wild-type and mutant mice at E18.5 were stained with monoclonal antibody (mAb) Ki-67, which is directed toward a nuclear protein expressed exclusively during the S, G_2 and M phase of the cell cycle (Schlütter *et al.*, 1993). To rule out effects caused by disrupted adhesion, we examined only non-lesional areas of mutant skin. As shown in Figure 7A, the epidermis of mutant mice contained significantly fewer Ki-67-positive cells per linear millimeter of basement membrane (33.7 ± 4.0) than that of wild-type mice (61.7 ± 8.1). In addition, in normal mice at E18.5, both the basal and immediately suprabasal layers contained a large fraction of Ki-67-positive cells, but in mutant mice cell proliferation was largely restricted to the basal layer (Figure 7A). This result indicates that the cytoplasmic domain of β4 is required for optimal proliferation of epidermal precursor cells.

The integrin α6β4 is also expressed in several simple epithelia including the gastrointestinal tract (Simon-Assmann *et al.*, 1994). The epithelium of the intestine is renewed continually from a small number of proliferating cells concentrated in invaginated structures named crypts. Proliferation ceases in the upper segment of the crypt, and post-mitotic enterocytes migrate toward the tip of the villus and begin to differentiate (Gordon and Hermiston, 1994). Different laminin variants are expressed along the crypt–villus axis: laminin 2 is enriched in the crypts, laminin 1 is more abundant in villi and laminin 5 is expressed in both (Simon-Assmann *et al.*, 1994; Orian-Rousseau *et al.*, 1996). Laminin 1 has been shown recently to contribute to the establishment and maintenance of differentiation in enterocyte-like CaCo2 cells (De Arcangelis *et al.*, 1996). The role of other laminin isoforms and their integrin receptors in intestinal function remains to be determined.

To determine if α6β4 signaling also regulated proliferation in simple epithelia, we focused on the intestine. In the proximal duodenum at E18.5, α6β4 and laminin 5 were concentrated at the basement membrane junction in both intervillar spaces and villi (data not shown), and cell proliferation was confined to the intervillar spaces, which invaginate later to form the crypts (Figure 7A). We observed that the total number of Ki-67-positive cells per intervillar space was lower in mutant mice (8.2 ± 1.4) than in wild-type mice (12.9 ± 3.0). In addition, while in normal mice at this stage of development the basal portion of most villi contained a large fraction of Ki-67-positive enterocytes, cell proliferation was largely restricted to the intervillar spaces in mutant mice. Since adhesion in these sections was intact, these findings indicate that signals transduced by the β4 tail are necessary to maintain a normal proliferative compartment in both the epidermis and intestine.

Previous studies have indicated that cells denied adhesion to the ECM, and thereby arrested in mid-G_1, contain elevated levels of cyclin-dependent kinase (cdk) inhibitors such as $p27^{Kip}$ (Fang *et al.*, 1996; Zhu *et al.*, 1996). We therefore stained sections of duodenum from wild-type and mutant mice with antibodies to $p27^{Kip}$. While in normal mice the enterocytes of intervillar spaces and the

basal portion of villi did not contain detectable levels of $p27^{Kip}$, in mutant mice the immunostaining for $p27^{Kip}$ extended to the base of villi with frequent $p27^{Kip}$-positive cells in the intervillar spaces (Figure 7B, arrows). Accordingly, the intervillar spaces of mutant mice contained fewer p27-negative cells (9.7 ± 2.6) than those of wild-type mice (15.6 ± 4.2). In addition, the nuclei of post-mitotic enterocytes in mutant mice were stained more intensely by anti-p27 antibodies than those in wild-type mice. Although the suprabasal layers of skin contain another cdk inhibitor, $p21^{Cip}$, we did not detect increased accumulation of this inhibitor in the suprabasal layers of mutant mice (data not shown). These results suggest that α6β4 signaling contributes to the regulation of $p27^{Kip}$, but not $p21^{Cip}$. Taken together, the reduced Ki-67 labeling and increased $p27^{Kip}$ staining in the intestine of mutant mice are consistent with the conclusion that α6β4 signaling is necessary for optimal proliferation *in vivo*.

Finally, we examined whether the reduction of progenitor cells associated with the tail-less B4 defect affected differentiation in the epidermis or intestine. Immunofluorescent analysis indicated that the pattern of expression of epidermal differentiation markers involucrin (data not shown), filaggrin and loricrin was similar in wild-type and mutant mice at E18.5 (Figure 8). In addition, assays of enzymatic activity *in situ* indicated that the level of expression and localization of lactase in the intestine were similar in wild-type and mutant mice at E18.5 (Figure 8). These results, together with those of histological analysis, suggest that α6β4 signaling is not required for normal differentiation of skin and intestine during embryogenesis.

Biological implications

It is well established that normal cells must adhere to the ECM in order to proliferate *in vitro* (Giancotti and Mainiero, 1994). The phenotype of mice lacking the β4 tail provides direct evidence that integrin signaling regulates the cell cycle *in vivo*. In particular, the observation that the number of proliferating cells in the basal layer of epidermis and intervillar space of intestine is significantly reduced in mutant mice, despite the presence of other Shc-linked integrins in these cells, suggests that the combined input from multiple Shc-linked integrins is required for optimal proliferation. Previous studies have indicated that the basal layer of human epidermis contains two types of precursor cells that can be identified on the basis of their β1 integrin expression levels: the slow-cycling stem cells, which express high levels of α3β1 and α2β1; and the more rapidly proliferating transit amplifying cells, which display lower levels of β1 integrins (Jones and Watt, 1993; Jones *et al.*, 1995). Notably, α6β4 is expressed at similar levels on both stem cells and transit-amplifying cells (Jones and Watt, 1993; Jones *et al.*, 1995), and may thus affect the proliferation of both types of precursor cells.

In accordance with the observation that anchorage to the ECM is required for proper down-regulation of the cdk inhibitor $p27^{Kip}$ in early-to-mid G_1 (Zhu *et al.*, 1996), we have found that in tail-less β4 mice the enterocytes located at the base of villi display increased nuclear levels of $p27^{Kip}$. In contrast, the post-mitotic keratinocytes did not contain increased levels of the other cdk inhibitor, $p21^{Cip}$, expressed in the epidermis. These observations suggest that increased levels of $p27^{Kip}$ in the intestinal precursor cells of mutant mice may contribute to the premature withdrawal from the cell cycle. However, the presence of a significant proliferation defect in the basal layer of the epidermis suggests that the down-regulation of cdk inhibitors is not the only mechanism by which integrin signaling promotes cell cycle progression. Other mechanisms, such as the ability of integrins and growth factor receptors to coordinately regulate the expression of immediate-early genes, including D-type cyclins, are likely to play a crucial role.

The proliferation defect observed in mice lacking the β4 tail does not result in a reduced production of differentiated cells in the skin and intestine of E18.5 embryos. This is not surprising because the precursor cell compartment in normal skin and intestine at this stage of development is much larger than in the adult, and thus presumably exceeds the physiological requirements of the tissues. Based on the approach illustrated here, it should now be possible to introduce in mice a β4 mutation that abolishes signaling without affecting linkage to the cytoskeleton, and thus to examine if α6β4 signaling is necessary for homeostasis of adult mouse skin and intestine.

It has been speculated widely that the association of integrins with the cytoskeleton is necessary to stabilize adhesion to the ECM (Alberts *et al.*, 1994). The phenotype of mice lacking the β4 tail provides a particularly vivid illustration of the importance of cytoskeletal anchorage for stable adhesion. Because of the association of β4 tail with the keratin cytoskeleton, stresses applied to the α6β4–laminin 5 bond may be distributed to the entire cytoskeleton. This view is consistent with the results of static and laminar flow adhesion assays and the observation that a significant fraction of tail-less α6β4 appears to be pulled out of the membrane when the epidermis of mutant mice detaches from the basement membrane.

The phenotype of tail-less β4 mice closely resembles that of humans affected by PA-JEB, including the blistering of the skin and the detachment and degeneration of the pyloric epithelium. Therefore, the cell adhesion defect in PA-JEB patients is most likely derived from the disruption of the transmembrane link between the basement membrane and keratin cytoskeleton mediated by α6β4. Our observation that α6β4 also regulates epidermal proliferation may explain another important feature of PA-JEB. In contrast to individuals with classical JEB, which have a severe form of skin blistering caused by mutations in the α6β4 ligand laminin 5, but never display areas of skin hypoplasia or aplasia (Aberdam *et al.*, 1994; Pulkkinen *et al.*, 1994), newborns affected by PA-JEB display extensive areas of skin aplasia, especially in the lower limbs (Vidal *et al.*, 1995; Ruzzi *et al.*, 1997). Since the production of differentiated keratinocytes appears to be normal in our mutant mice, it is possible that the precursor cell compartment in certain areas of human fetal skin is smaller than in mice so that the α6β4 mutation results in neonatal aplasia only in humans.

In conclusion, the results of this study indicate that the intracellular interactions mediated by the β4 cytoplasmic domain are necessary for stable adhesion of stratified epithelia to the basement membrane and for proper cell cycle control in both simple and stratified epithelia. These findings reveal that normal cells are also exquisitely

anchorage-dependent *in vivo* and help to understand the molecular basis of the human blistering skin disease PA-JEB.

Materials and methods

Targeted deletion of the β4 cytoplasmic domain

A 7 kb fragment of the mouse gene β4 was isolated by screening a 129Sv library with a human cDNA probe complementary to the sequence encoding the transmembrane domain. Site-directed mutagenesis was used to introduce a stop codon followed by a novel *Eco*RI site immediately after the sequence encoding the transmembrane domain. The *Eco*RI site was then used to subclone an SV40 polyadenylation signal followed by a neomycin resistance expression cassette downstream of the stop codon. Finally, the modified fragment was introduced into the targeting vector pPNT (Tybulewicz *et al.*, 1991) in two steps (left arm 5 kb and right arm 1.8 kb). Prior to electroportation in ES cells (Swiatek and Gridley, 1993), the targeting vector was linearized by digestion with *Not*I. Positively transfected cells which had undergone homologous recombination were selected in 0.5 mg/ml G418 and 0.2 mM gancyclovir and identified by Southern blotting. Six distinct ES cell lines were found to carry the expected mutation, and four were injected into blastocyst-stage C57BL/6 mouse embryos which were then transplanted into the uteri of pseudopregnant C57BL/6 mice. Two lines produced extensively chimeric male mice which were crossed to C57BL/6 females. Heterozygous offspring were then used to generate embryos homozygous for the targeted deletion. Embryos were genotyped by PCR using genomic DNA isolated from the forelimb. The primers used for amplification were: right arm primer, 5′-GATCTTCCAGCGGACTGTGTC-3′; neomycin cassette primer, 5′-GCTCCCGATTCGCAGCGCCATCG-3′; and wild-type specific primer, 5′-AGAGGATGGTGTCCTCCTCA-3′. The wild-type allele yielded a 0.5 kb fragment and the mutant allele gave rise to a 0.8 kb fragment.

Northern blotting

Total RNA was extracted from mouse skin and hybridized to a 0.5 kb cDNA probe complementary to sequences in the β4 ectodomain. The probe was generated by amplifying reverse-transcribed RNA from mouse PAM keratinocytes with the following primers: 5′-CTGTGAGCAAGGAAGTT-3′ and 5′-CGTGTAGAGCGACTGCTGGTT-3′.

Antibodies

The rabbit antiserum SE144 recognizes the γ2 subunit of laminin 5 (Vailly *et al.*, 1994) and mAb 4E10 the β2 subunit of laminin 1, 2, 6 and 8 (Engvall *et al.*, 1986). The rat anti-mouse α6 mAb GoH3 and hamster anti-mouse β1 mAb HMβ1-1 were purchased from Pharmingen (Los Angeles, CA). The rabbit antiserum to a GST fusion protein comprising amino acids 31–217 of human β4 has been described (Maniero *et al.*, 1997). mAb 346-11A reacts with the ectodomain of mouse β4 (Kennel *et al.*, 1989). The antibodies to BPAG2 were raised by immunizing rabbits with a GST fusion protein comprising amino-acids 1–250 of mouse BPAG2 and affinity purified on the antigen. MAb5E reacts with BPAG1 (Shimizu *et al.*, 1991) and mAb 121 with HD1/plectin (Hieda *et al.*, 1992). Affinity-purified rabbit antibodies to synthetic peptides from mouse filaggrin, loricrin, keratin 1, keratin 5 and involucrin were characterized previously (Calautti *et al.*, 1995). The anti-Ki-67 mAb MM1 was produced at the Monoclonal Antibody Facility of Sloan Kettering Institute. Affinity-purified antibodies to a GST fusion protein comprising full-length $p27^{Kip}$ were described previously (Casaccia-Bonnefil *et al.*, 1997). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunoprecipitation

Skins from wild-type and homozygous mutant E18.5 embryos were extracted in RIPA buffer containing protease inhibitors. Total epidermal proteins were normalized by immunoblotting with anti-keratin 1 antibodies. Equivalent amounts of total epidermal proteins were immunoprecipitated with the anti-α6 mAb GoH3 and subjected to immunoblotting with the antiserum to the β4 ectodomain (Mainiero *et al.*, 1997).

Adhesion assays

Primary mouse keratinocytes were isolated as described (DiPersio *et al.*, 1997). For static adhesion assay, primary keratinocytes from wild-type and mutant E18.5 embryos were pre-incubated with 10 μg/ml of anti-β1 mAb HMβ1-1, to block the adhesive function of α3β1, and plated for 30 min in the presence of the same antibody on microtiter wells coated with the indicated concentrations of purified laminin 5. For laminar flow adhesion assay (Brieher *et al.*, 1996), primary keratinocytes from wild-type and mutant E18.5 embryos were pre-incubated with 10 μg/ml anti-β1 mAb HMβ1-1 and drawn into a glass capillary coated with 10 μg/ml purified laminin 5. The cells were allowed to bind under static conditions for 30 min in the presence of the same antibody and then subjected to increasing flow rates. The number of cells remaining adherent after each step was evaluated microscopically.

Immunohistochemistry and lactase assay

Skin and intestine samples were harvested from embryos and embedded in either OCT or paraffin. Tissue sections (10 μM) were stained with hematoxylin and eosin or subjected to either immunofluorescence or imunoperoxidase staining. Lactase activity was assayed on tissue sections as previously described (Stoward and Everson Pearse, 1991).

Acknowledgements

We thank V.Soares and W.Mark of the Transgenic Mouse Facility, K.Manova of the Molecular Citology Facility, and N.Lampen of the Electron Microscopy Facility for their invaluable help; E.Calautti, G.P.Dotto and C.M.DiPersio for their advice on the isolation of primary keratinocytes; W.M.Brieher for assistance in the laminar flow assays; P.Rousselle and R.E.Burgeson for providing purified laminin 5; E.Colautti, G.P.Dotto, E.Engvall, T.Hashimoto, S.J.Kennel, A.Koff, G.Meneguzzi, K.Owaribe, M.Park and F.Watt for antibodies; and C.Blobel and B.Gumbiner for comments on the manuscript and discussions. This work was supported by DAMD grant 17-94-J4306 and NIH grants R01-CA58976 and P30-CA08748. C.M. was on leave of absence from the Istituto Nazionale della Nutrizione in Rome. F.G.G. is an Established Investigator of the American Heart Association.

References

Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K. and Watson,J.D. (1994) Extracellular matrix receptors on animal cells: the integrins. In *Molecular Biology of the Cell*. 3rd edn. Garland Publishing, Inc., New York and London, pp. 995–999.

Aberdam,D. *et al.* (1994) Herlitz's junctional epidermolysis bullosa is genetically linked to mutations in the nicein/kalinin (laminin-5) LAMC2 gene. *Nature Genet.*, **6**, 299–304.

Borradori,L. and Sonnenberg,A. (1996) Hemidesmosomes: roles in adhesion, signaling and human diseases. *Curr. Opin. Cell Biol.*, **8**, 647–656.

Borradori,L., Koch,P.J., Niessen,C.M., Erkeland,S. and van Leusden,M.R. (1997) The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the β4 integrin subunit. *J. Cell Biol.*, **136**, 1333–1347.

Brieher,W.M., Yap,A.S. and Gumbiner,B.M. (1996) Lateral dimerization is required for the homophilic binding activity of C-cadherin. *J. Cell Biol.*, **135**, 487–496.

Burridge,K. and Chrzanowska-Wonidka,M. (1996) Focal adhesions, contractility, and signaling. *Annu. Rev. Dev. Biol.*, **12**, 463–519.

Calautti,E., Missero,C., Stein,P.L., Ezzel,R.M. and Dotto,G.P. (1995) Fyn tyrosine kinase is involved in keratinocyte differentiation control. *Genes Dev.*, **9**, 2279–2291.

Carter,W.G., Kaur,P., Gil,S.G., Gahr,P.J. and Wayner,E.A. (1990) Distinct functions for integrins α3β1 in focal adhesions and α6β4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.*, **111**, 3141–3154.

Carter,W.G., Ryan,M.C. and Gahr,P.J. (1991) Epiligrin, a new cell adhesion ligand for integrin α3β1 in epithelial basement membranes. *Cell*, **65**, 599–610.

Casaccia-Bonnefil,P., Tikoo,R., Kiyokawa,H., Friedrich,V.,Jr, Chao,M.V. and Koff,A. (1997) Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27Kip1. *Genes Dev.*, **11**, 2335–2346.

De Arcangelis,A., Neuville,P., Boukamel,R., Lefebvre,O., Kedinger,M. and Simon-Assmann,P. (1996) Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation. *J. Cell Biol.*, **133**, 417–430.

DiPersio,C.M., Hodivala-Dilke,K.M., Jaenisch,R., Kreidberg,J.A. and Hynes,R.O. (1997) α3β1 integrin is required for normal development of the epidermal basement membrane. *J. Cell Biol.*, **137**, 729–742.

Dowling,J., Yu,Q.-C. and Fuchs,E. (1996) β4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.*, **134**, 559–572.

Einheber,S., Milner,T.A., Giancotti,F.G. and Salzer,J.L. (1993) Axonal regulation of Schwann cell integrin expression suggests a role for α6β4 in myelination. *J. Cell Biol.*, **123**, 1223–1236.

Engvall,E., Davis,G.E., Dickerson,K., Ruoslahti,E., Varon,S. and Manthorpe,M. (1986) Mapping of domains in human laminin using monoclonal antibodies: localization of the neurite-promoting site. *J. Cell Biol.*, **103**, 2457–2465

Fang,F., Orend,G., Watanabe,N., Hunter,T. and Ruoslahti,E. (1996) Dependence of cyclin E–CDK 2 kinase activity on cell anchorage. *Science*, **271**, 499–502.

Gandarillas,A. and Watt,F.M. (1997) c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.*, **11**, 2869–2882.

Georges-Labouesse,E., Messaddeq,N., Yehia,G., Cadalbert,L., Dierich,A. and Le Meur,M. (1996) Absence of integrin α6 leads to epidermolysis bullosa and neonatal death in mice. *Nature Genet.*, **13**, 370–373.

Giancotti,F.G. (1996) Signal transduction by the α6β4 integrin: charting the path between laminin binding and nuclear events. *J. Cell Sci.*, **109**, 1165–1172.

Giancotti,F.G. (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.*, **9**, 691–700.

Giancotti,F.G. and Mainiero,F. (1994) Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta*, **1198**, 47–64.

Giancotti,F.G., Stepp,M.A., Suzuki,S., Engvall,E. and Ruoslahti,E. (1992) Proteolytic processing of endogenous and recombinant β4 integrin subunit. *J. Cell Biol.*, **118**, 951–959.

Gordon,J.I. and Hermiston,M.L. (1994) Differentiation and self-renewal in the mouse gastrointestinal epithelium. *Curr. Opin. Cell Biol.*, **6**, 795–803.

Green,H. (1977) Terminal differentiation of cultured human epidermal cells. *Cell*, **11**, 405–416.

Guo,L., Degenstein,L., Dowling,J., Yu,Q.-C., Wollmann,R., Perman,B. and Fuchs,E. (1995) Gene targetting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. *Cell*, **81**, 233–243.

Hall,P.A. and Watt,F.M. (1989) Stem cells: the generation and maintenance of cellular diversity. *Development*, **106**, 619–633.

Hemler,M.E. (1990) VLA proteins in the integrin family. *Annu. Rev. Immunol.*, **8**, 365–400.

Hieda,Y., Nishizawa,Y., Uematsu,J. and Owaribe,K. (1992) Identification of a new hemidesmosomal protein, HD1: a major high molecular mass component of isolated hemidesmosomes. *J. Cell. Biol.*, **116**, 1497–1506.

Hogervorst,F., Kuikman,I., von dem Borne,A.E.G.Kr. and Sonnenberg,A. (1990) Cloning and sequence analysis of β4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J.*, **9**, 745–770.

Hynes,R.O. (1987) Integrins: a family of cell surface receptors. *Cell*, **48**, 549–554.

Jones,P.H. and Watt,F.M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell*, **73**, 713–724.

Jones,P.H., Harper,S. and Watt,F.M. (1995) Stem cell patterning and fate in human epidermis. *Cell*, **80**, 83–93.

Kajiji,S., Tamura,R.N. and Quaranta,V. (1989) A novel integrin (αEβ4) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.*, **8**, 673–680.

Kennel,S.J., Foote,L.J., Falcioni,R., Sonnenberg,A., Stringer,C.D., Crouse,C. and Hemler,M.E. (1989) Analysis of the tumor-associated antigen TSP-180. Identity with alpha 6-beta-4 in the integrin superfamily. *J. Biol. Chem.*, **264**, 15515–15521.

Kennel,S.J., Godfrey,V., Chang,L.Y., Lankford,T.K., Foote,L.J. and Makkinje,A. (1992) The β4 subunit of the integrin family is displayed on a restricted subset of endothelium in mice. *J. Cell Biol.*, **101**, 145–150.

Kimmel,K.A. and Carey,T.E. (1986) Altered expression in squamuos carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.*, **46**, 3614–2623.

Klein,S., Giancotti,F.G., Presta,M., Albelda,S.M., Buck,C.A. and Rifkin,D.B. (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell*, **4**, 973–982.

Mainiero,F., Pepe,A., Wary,K.K., Spinardi,L., Mohammadi,M., Schlessinger,J. and Giancotti,F.G. (1995) Signal transduction by the α6β4 integrin: distinct β4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO J.*, **14**, 4470–4481.

Mainiero,F., Murgia,C., Wary,K.K., Curatola,A.M., Pepe,A., Blumemberg,M., Westwick,J.K., Der,C.J. and Giancotti,F.G. (1997) The coupling of α6β4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J.*, **16**, 2365–2375.

Niessen,C.M., Hulsman,E.H.M., Rots,E.S., Sanchez-Aparicio,P. and Sonnenberg,A. (1997) Integrin α6β4 forms a complex with the cytosleletal protein HD1 and induces its redistribution in transfected COS-7 cells. *Mol. Biol. Cell*, **8**, 555–566.

Orian-Rousseau,V., Aberdam,D., Fontao,L., Chevalier,L., Meneguzzi,G., Kedinger,M. and Simon-Assmann,P. (1995) Developmental expression of laminin-5 and HD1 in the intestine: epithelial to mesenchymal shift for the laminin γ2 chain subunit deposition. *Dev. Dynam.*, **206**, 12–23.

Pulkkinen,L., Christiano,A.M., Airenne,T., Haakana,H., Tryggvason,K. and Uitto,J. (1994) Mutations in the γ2 chain gene (LAMC2) of kalinin/laminin 5 in the junctional forms of epidermolysis bullosa. *Nature Genet.*, **6**, 293–298.

Reznieczek,G.A., de Pereda,J.M., Reipert,S. and Wiche,G. (1998) Linking integrin α6β4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the β4 subunit and plectin at multiple molecular sites. *J. Cell Biol*, **141**, 209–225.

Rousselle,P., Lunstrum,G.P., Keene,D.R. and Burgeson,R.E. (1991) Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.*, **114**, 567–576.

Ruoslahti,E. and Pierschbacher,D. (1987) New perspectives in cell adhesion: RGD and integrins. *Science*, **238**, 491–497.

Ruzzi,L., Gagnoux-Palacios,L., Pinolaq,M., Belli,S., Meneguzzi,G., D'Alessio,M.D. and Zambruno,G.A. (1997) Homozygous mutation in the integrin α6 gene in junctional epidermolysis bullosa with pyloric atresia. *J. Clin. Invest.*, **99**, 2826–2831.

Schlütter,C., Duchrow,M., Wohlenberg,C., Becker,M.H.G., Key,G. Flad,H.-D. and Gerdes,J. (1993) The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining protein. *J. Cell Biol.*, **123**, 513–522.

Shaw,L.M., Rabinovitz,I., Wang,H., Toker,A. and Mercurio,A.M. (1997) Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. *Cell*, **91**, 949–960.

Shimizu,H., Hashimoto,T. and Eady,R.A. (1991) Human monoclonal anti basement membrane zone antibodies derived from virally transformed lymphocytes of a patient with bullous pemphigoid recognize epitopes associated with hemidesmosomes. *Br. J. Dermatol.*, **124**, 217–220.

Simon-Assmann,P., Duclos,B., Orian-Rousseau,V., Arnold,C., Mathelin,C., Engvall,E. and Kedinger, M. (1994) Differential expression of laminin isoforms and α6-β4 integrin subunits in the developing human and mouse intestine. *Dev. Dynam.*, **201**, 71–85.

Sonnenberg,A., Linders,C.J.T., Daams,J.H. and Kennel,S.J. (1990) The α6β1 (VLA-6) and α6β4 protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.*, **96**, 207–217.

Spinardi,L., Ren,Y.-L., Sanders,R. and Giancotti,F.G. (1993) The β4 subunit cytoplasmic domain mediates the interaction of α6β4 integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell*, **4**, 871–884.

Spinardi,L., Einheber,S., Cullen,T., Milner,T.A. and Giancotti,F.G. (1995) A recombinant tail-less integrin β4 subunit disrupts hemidesmosomes, but does not suppress α6β4-mediated cell adhesion to laminins. *J. Cell Biol.*, **129**, 473–487.

Stepp,M.A., Spurr-Michaud,S., Tisdale,A., Elwell,J. and Gipson,I.K. (1990) Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl Acad. Sci. USA*, **87**, 8970–8974.

Stoward,P.J. and Everson Pearse,A.G. (1991) *Histochemistry*. Churchill Livingston, Edinburgh, UK, Vol. 3, pp. 637–638.

Suzuki,S. and Naitoh,Y. (1990) Amino acid sequence of a novel integrin β4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.*, **9**, 757–763.

Swiatek,P.J. and Gridley,T. (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger protein Krox20. *Genes Dev.*, **7**, 2071–2084.

Timpl,R. and Brown,J.C. (1996) Supramolecular assembly of basement membranes. *BioEssays*, **18**, 123–132.

Tybulewicz,V.L., Crawford,C.E., Jackson,P.K., Bronson,R.T. and Mulligan,R.C. (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. *Cell*, **65**, 1153–1563.

Vailly,J. *et al.* (1994) The 100-kDa chain of nicein/kalinin is a laminin B2 chain variant. *Eur. J. Biochem.*, **219**, 209–218.

van der Neut,R., Krimpenfort,P., Calafat,J., Niessen,C.M. and Sonnenberg,A. (1996) Epithelial detachment due to absence of hemidesmosomes in integrin β4 null mice. *Nature Genet.*, **13**, 366–369.

Vidal,F., Aberdam,D., Miquel,C., Christiano,A.M., Pulkkinen,L., Uitto,J., Ortonne,J.P. and Meneguzzi, G. (1995) Integrin β4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nature Genet.*, **10**, 229–234.

Yurchenco,P.D. and O'Rear,J.J. (1994) Basal lamina assembly. *Curr. Opin. Cell Biol.*, **6**, 674–681.

Wadsworth,S., Halvorson,M.J. and Coligan,J.E. (1992) Developmentally regulated expression of the β4 integrin on immature mouse thymocytes. *J. Immunol.*, **149**, 421–428.

Wary,K.K., Mainiero,F., Isakoff,S.J., Marcantonio,E.E. and Giancotti,F.G. (1996) The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell*, **87**, 733–743.

Wolf,G.T., Carey,T.E., Schmaltz,S.P., McClatchey,K.D., Poore,J., Glaser,L., Hayashida,D.J.S. and Hsu,S. (1990) Altered antigen expression predicts outcome in squamous cell carcinomas of the head and neck. *J. Natl Cancer Inst.*, **82**, 1566–1572.

Zhu,X., Ohtsubo,M., Bohmer,R., Roberts,J.M. and Assoian,R.K. (1996) Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E–cdk2, and phosphorylation of the retinoblastoma protein. *J. Cell Biol.*, **133**, 391–403.

Received March 5, 1998; revised and accepted May 12, 1998

Note added in proof

A recent study provides further evidence that integrin-mediated Shc signalling regulates cell proliferation *in vivo* [Pozzi,A., Wary,K.K., Giancotti,F.G. and Gardner,H. (1998) Integrin α1β1 mediates a unique collagen dependent proliferation pathway *in vivo*. *J. Cell Biol.*, in press].

Cell, Vol. 94, 625–634, September 4, 1998, Copyright ©1998 by Cell Press

A Requirement for Caveolin-1 and Associated Kinase Fyn in Integrin Signaling and Anchorage-Dependent Cell Growth

Kishore K. Wary,* Agnese Mariotti,*
Chiara Zurzolo,† and Filippo G. Giancotti*‡
*Cellular Biochemistry and Biophysics Program
Memorial Sloan-Kettering Cancer Center
New York, New York 10021
†Dipartimento di Biologia e Patologia Cellulare e Molecolare
University of Naples
Naples 80131
Italy

Summary

Caveolin-1 functions as a membrane adaptor to link the integrin α subunit to the tyrosine kinase Fyn. Upon integrin ligation, Fyn is activated and binds, via its SH3 domain, to Shc. Shc is subsequently phosphorylated at tyrosine 317 and recruits Grb2. This sequence of events is necessary to couple integrins to the Ras–ERK pathway and promote cell cycle progression. These findings reveal an unexpected function of caveolin-1 and Fyn in integrin signaling and anchorage-dependent cell growth.

Introduction

For proper embryonic development, tissue homeostasis, and wound healing, cell proliferation must be tightly regulated, both in space and over time. In particular, a cell must be able to sense its relationship to other cells and the extracellular matrix and convert these positional cues into biochemical signals affecting the cell cycle. Because of their ability to couple the recognition of positional cues to the activation of intracellular signaling pathways, adhesion receptors are likely to be necessary to achieve this goal.

Integrins mediate cell adhesion primarily by binding to distinct, although overlapping, subsets of extracellular matrix proteins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Normal cells need to adhere to serum-derived extracellular matrix components such as fibronectin and vitronectin in order to proliferate in vitro, a phenomenon called anchorage dependence. By contrast, neoplastic cells do not require adhesion for growth (reviewed in Giancotti and Mainiero, 1994). It naturally follows that integrins must provide cells with signals that are necessary for the proliferation of normal, but not neoplastic, cells.

There are clear indications that the extracellular matrix can promote either proliferation or growth arrest and differentiation. The outcome appears to be dictated by the composition of the extracellular matrix and the repertoire of integrins on the cell, and only to a lesser degree by the extent of its cytoskeletal organization (Giancotti, 1997). The simplest hypothesis is that a class of integrins activate a biochemical pathway necessary for cell cycle progression, while other integrins are unable to do so.

Recent studies have revealed that integrins activate common as well as subgroup-specific signaling pathways (Clark and Hynes, 1997; Giancotti, 1997). In particular, while most integrins activate focal adhesion kinase (FAK), α1β1, α5β1, αvβ3, and α6β4 are coupled to the Ras-extracellular signal-*r*egulated *k*inase (ERK) signaling pathway by Shc (Mainiero et al., 1995, 1997; Wary et al., 1996). Shc is an SH2-PTB domain adaptor protein that links various tyrosine kinases to Ras by recruiting the Grb2/SOS complex to the plasma membrane (Pawson and Scott, 1997). Upon activation by SOS, Ras stimulates a kinase cascade, culminating in the activation of the mitogen-activated protein kinase (MAPK) ERK (Marshall, 1995). ERK phosphorylates ternary complex transcription factors such as Elk-1 and Sap-1/2 and promotes transcription of the immediate-early gene *Fos* (Treisman, 1996). In primary endothelial cells and keratinocytes, mitogens and Shc-linked integrins cooperate, in a synergic fashion, to promote transcription from the *Fos* promoter. Accordingly, ligation of integrins linked to Shc enables these cells to progress through G1 in response to mitogens, whereas ligation of other integrins results in growth arrest, even in the presence of mitogens (Wary et al., 1996; Mainiero et al., 1997). These results suggest a direct role of integrin-dependent Shc signaling in anchorage-dependent cell growth.

Previous studies have suggested that the recruitment of Shc by activated β1 and αv integrins is indirect and possibly mediated by caveolin-1 (Wary et al., 1996). Caveolin-1 is the prototype of a family of small proteins forming a hairpin structure in the plasma membrane, with both N- and C-terminal domains facing the cytoplasm. A fraction of caveolin-1 forms high-molecular-weight homooligomers and heterooligomers with caveolin-2 in the ER. After transport to the Golgi apparatus, the oligomers increase in size and become insoluble in Triton X-100 due to interactions with glycosphingolipid/cholesterol–enriched domains (rafts). The resulting structures may participate in the biogenesis of post-Golgi transport vesicles and flask-shaped plasma membrane invaginations called caveolae. Although the function of caveolae is not clear, they may participate in intracellular transport and possibly assembly of signaling complexes (Harder and Simons, 1997; Okamoto et al., 1998).

The results reported here indicate that a Triton X-100 soluble fraction of caveolin-1 physically and functionally links integrins to Fyn. Upon activation, this kinase recruits Shc and thereby regulates Ras–ERK signaling and cell cycle progression.

Results

Integrins Associate with a Triton X-100 Soluble Fraction of Caveolin-1

The role of caveolin-1 in integrin signaling was examined in normal, untransformed cells because the expression of caveolin-1 (Koleske et al., 1995) and of integrins α1β1

‡To whom correspondence should be addressed.

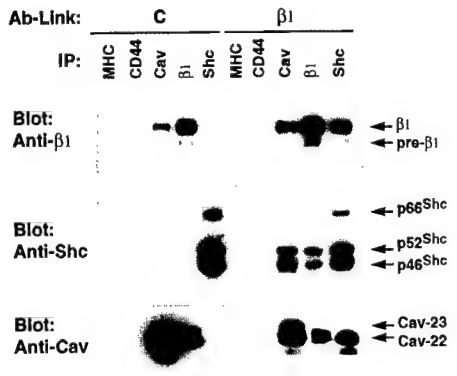


Figure 1. Coimmunoprecipitation of β1 Integrins with Caveolin-1 and Shc

WI-38 cells were incubated in suspension with polystyrene beads coated with control anti-MHC MAb W6.32 ("C") or anti-β1 integrin MAb 4B4 (β1) for 5 min and extracted with a buffer containing 1% Triton X-100. Equal amounts of total proteins were immunoprecipitated with MAb W6.32 (MHC), P1G12 (CD44), CO60 (Cav), TS2/16 (β1), or PG-797 (Shc) and probed by immunoblotting with a rabbit antiserum to the β1 cytoplasmic domain (top), affinity-purified antibodies to the Shc SH2 domain (middle), or affinity-purified antibody C13630 to the N-terminal cytoplasmic domain of caveolin-1 (bottom).

and α5β1 (Plantefaber and Hynes, 1989), which are linked to Shc signaling, is often reduced in neoplastic cells. WI-38 fibroblasts were incubated with polystyrene beads coated with control or anti-β1 integrin MAbs and solubilized with Triton X-100 under conditions that preserve the association of activated integrins with Shc but do not solubilize caveolae. As shown in Figure 1, the anti-caveolin-1 antibodies coimmunoprecipitated a fraction of β1 integrins from both control and anti-β1 stimulated cells. Conversely, the anti-β1 antibodies coprecipitated a fraction of caveolin-1. Upon integrin cross-linking, the anti-caveolin-1 and anti-integrin antibodies also coimmunoprecipitated Shc. In addition, the anti-Shc antibodies coimmunoprecipitated both caveolin-1 and β1 integrins. These results indicate that a fraction of β1 integrins and caveolin-1 form a complex independently of integrin ligation and that this complex combines with Shc in response to integrin ligation. Typically, 20%–30% of the Triton X-100 soluble β1 integrins coprecipitated with caveolin-1 and vice versa. Because of the disruptive nature of solubilization with detergent, these numbers probably underestimate the stoichiometry of the association in vivo. Since the oligomeric fraction of caveolin-1 is insoluble in Triton X-100 (Monier et al., 1995), these results imply that β1 integrins combine with caveolin-1 and Shc outside caveolae.

Mesenchymal cells form two types of integrin-dependent adhesions in culture: the focal adhesions, which link integrins to the ends of the actin stress fibers and contain vinculin, talin, and FAK, and the extracellular matrix contacts, which associate laterally with stress fibers, contain the bulk of β1 integrins and extracellular fibronectin fibrils, but lack vinculin, talin, and FAK (Chen et al., 1985; Burridge and Chrzanowska-Wodnicka, 1996). To examine if caveolin-1 colocalized with integrins, WI-38 fibroblasts were subjected to correlative immunofluorescent staining. The results indicated that a significant fraction of caveolin-1 codistributes with integrins at extracellular matrix contacts, but not focal adhesions (unpublished). The subcellular localization of caveolin-1 confirms its association with integrins and suggests that Shc and FAK signaling may be topologically separated in cells.

The Transmembrane Domain of Integrin α Subunit Mediates Association with Caveolin-1 and Signaling via Shc

The functional significance of the association of integrins with caveolin-1 was explored by mutagenesis. Previous studies had indicated that a mutant single-chain tailless α1 subunit activates Shc signaling to ERK as efficiently as wild-type α1β1 (Wary et al., 1996). Chimeras of this mutant α1 subunit and the IL-2 receptor (IL-2R) α chain (Figure 2A) were introduced in NIH-3T3 fibroblasts by transient transfection. Immunoprecipitation and immunoblotting experiments indicated that the mutant α1 subunit associates with caveolin-1 and, upon antibody-mediated cross-linking, causes efficient recruitment of Shc and activation of ERK (construct A; Figures 2B and 2C). Replacement of the transmembrane segment of mutant α1 with the transmembrane and cytoplasmic portion of IL2-R α chain simultaneously abolished association with caveolin-1, recruitment of Shc, and activation of ERK (construct B; Figures 2B and 2C). This indicates that the integrin α subunit and caveolin-1 interact predominantly within the lipid bilayer.

Contrary to our prediction, a mutant consisting of the extracellular portion of IL2-R α chain linked to the transmembrane segment of α1 was not able to combine with caveolin-1 and mediate Shc signaling (not shown). However, another mutant identical to the above, but also containing the juxtamembrane region of the ectodomain of α1, did associate with caveolin-1 and cause efficient Shc signaling to ERK (construct C; Figures 2B and 2C). Since the direct adjoining of IL2-R α chain may disrupt the proper conformation of the transmembrane portion of α1, it remains possible that this portion of the integrin α subunit is sufficient for association with caveolin-1. Alternatively, the membrane proximal segment of the α subunit ectodomain may contribute to the interaction with caveolin-1, perhaps by associating with a necessary protein or lipid cofactor. Irrespective of the details of the integrin–caveolin-1 interaction, these results clearly indicate that the same limited segment of the integrin α subunit mediates both association with caveolin-1 and signaling via Shc.

Caveolin-1 Is Required for Integrin-Dependent Shc–Ras–ERK Signaling

The role of caveolin-1 in integrin-mediated signaling was directly tested by examining the caveolin-1-negative Fisher rat thyroid (FRT) cells and their counterpart cells stably transfected with a *caveolin-1* cDNA. The FRT cells express moderate levels of α6β4 that can recruit Shc directly (Mainiero et al., 1995, 1997), but very low or undetectable levels of the β1 and αv integrins, which recruit Shc by an indirect mechanism. The two cell lines

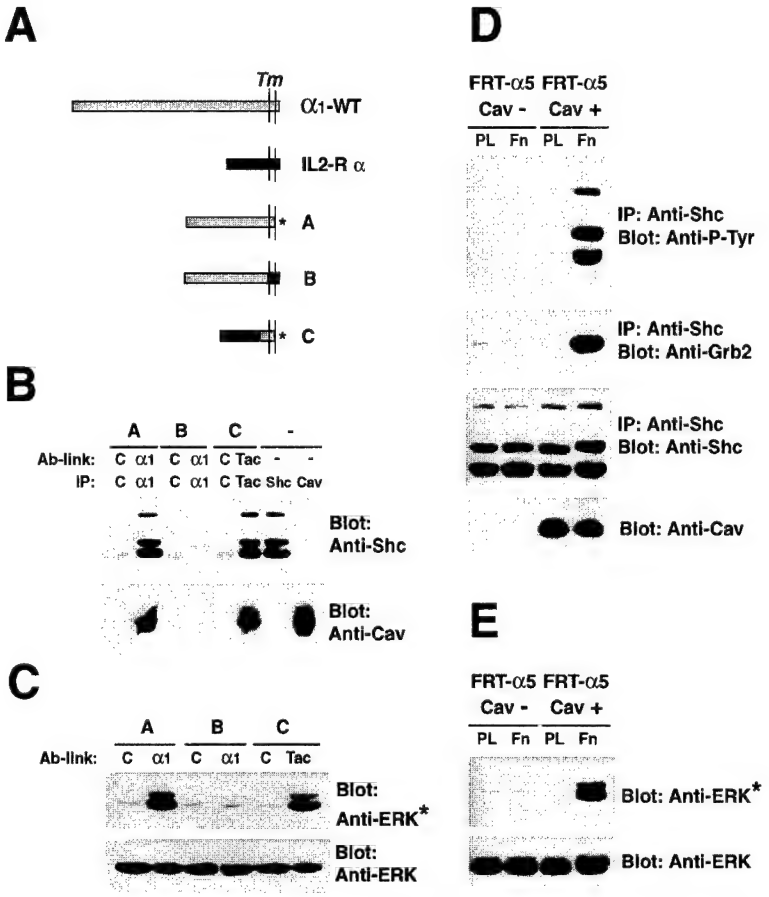


Figure 2. The Interaction of Integrin α Subunit with Caveolin-1 Is Required for Recruitment of Shc and Activation of ERK

(A) Schematic representation of wild-type integrin α1 subunit (α1-WT), IL-2R α chain, mutant α1 lacking the N-terminal 538 residues segment and entire cytoplasmic tail ("A"), chimera consisting of the extracellular portion of "A" fused to the transmembrane segment and cytoplasmic tail of IL-2R α ("B"), and chimera consisting of the IL-2R α ectodomain linked to the juxtamembrane and transmembrane domain of "A" ("C"). Asterisks point to deleted cytoplasmic domains.

(B) NIH-3T3 cells were transiently transfected with vectors encoding "A," "B," and "C", or empty vector (−). Aliquots were subjected to FACS analysis to verify that all recombinant proteins were expressed at similar levels. After 5 min of cross-linking with control MAb W6.32 ("C"), anti-α1 MAb TS2/7 (α1), or anti-IL-2R α MAb 4E3 (Tac), the cells transfected with "A," "B," and "C" were lysed with 1% Triton X-100, and equal amounts of total proteins were immunoprecipitated with the same antibodies used for cross-linking. Smaller aliquots of total extracts from cells transfected with empty vector were immunoprecipitated with anti-Shc MAb PG-797 (Shc) or anti-caveolin-1 MAb CO60 (Cav). The top portion of the blot was probed with affinity-purified rabbit antibodies to Shc and the bottom with the anti-caveolin-1 antibody C13630.

(C) Equal amounts of total proteins from NIH-3T3 cells transfected with vector encoding "A," "B," and "C" and cross-linked as above for 10 min were subjected to immunoblotting with anti-phospho-ERK (top). As a control, the blot was stripped and reprobed with a polyclonal antibody to ERK-2 (bottom). Asterisk shows anti-phospho-ERK.

(D) Parental and caveolin-1-expressing FRT cells were transiently transfected with a vector encoding human α5. Transfected cells were selected by panning on fibronectin, subjected to FACS analysis to verify that they expressed similar surface levels of α5β1, and replated on dishes coated with poly-L-lysine (PL) or fibronectin (Fn) for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc polyclonal antibodies and probed by immunoblotting with anti-p-Tyr MAb RC-20H (top) or anti-Grb2 polyclonal antibody (second panel). The top blot was stripped and reprobed with anti-Shc antibodies (third panel). As a control, total lysates were probed with affinity-purified antibody C13630 to caveolin-1 (bottom).

(E) Equal amounts of total proteins from parental and caveolin-1-expressing FRT cells, transfected with a vector encoding human α5, were plated either on poly-L-lysine (PL) or fibronectin (Fn) for 40 min and subjected to immunoblotting with anti-phospho-ERK (top). As a control, the blot was stripped and reprobed with a polyclonal antibody to ERK-2 (bottom). Asterisk shows anti-phospho-ERK.

were therefore transiently transfected with a vector encoding a human wild-type α5 subunit. Cells expressing comparable levels of α5β1 at the cell surface were plated on fibronectin or poly-L-lysine. As shown in Figure 2, adhesion to fibronectin caused tyrosine phosphorylation of Shc, association of Shc with Grb2, and activation of ERK in the caveolin-1-positive, but not -negative, FRT cells (Figures 2D and 2E). Identical results were obtained with two distinct FRT clones expressing similar levels of recombinant caveolin-1. These findings provide direct evidence that caveolin-1 is essential for Shc–Ras–ERK signaling in response to integrin ligation.

Caveolin-1 Associates with Fyn

To investigate the mechanism by which caveolin-1 mediates the recruitment and tyrosine phosphorylation of Shc, we searched for the responsible tyrosine kinase. Previous studies had suggested that caveolin-1 may interact with Src family kinases (Okamoto et al., 1998). To examine if the Triton X-100 soluble fraction of caveolin-1 associates with Src or Fyn, WI-38 fibroblasts were stimulated with control or anti-β1 antibodies, extracted with 1% Triton X-100, and subjected to coimmunoprecipitation analysis. As shown in Figure 3A, the anti-Fyn, but not anti-Src, antibodies coimmunoprecipitated a significant and similar fraction of caveolin-1 from both control and anti-β1 stimulated cells. Conversely, the anti-caveolin-1 antibodies coimmunoprecipitated Fyn, but not Src (Figure 3B). Upon overexpression in 293-T cells, however, both Src and Fyn coimmunoprecipitated with caveolin-1 (data not shown). These observations indicate that caveolin-1 is constitutively associated with Fyn, but not Src, in cells expressing physiological levels of these kinases.

Since it has been reported that caveolin-1 can also interact in vitro with the EGF receptor, Ras, and heterotrimeric G proteins (Okamoto et al., 1998), we examined whether the Triton X-100 soluble fraction of caveolin-1

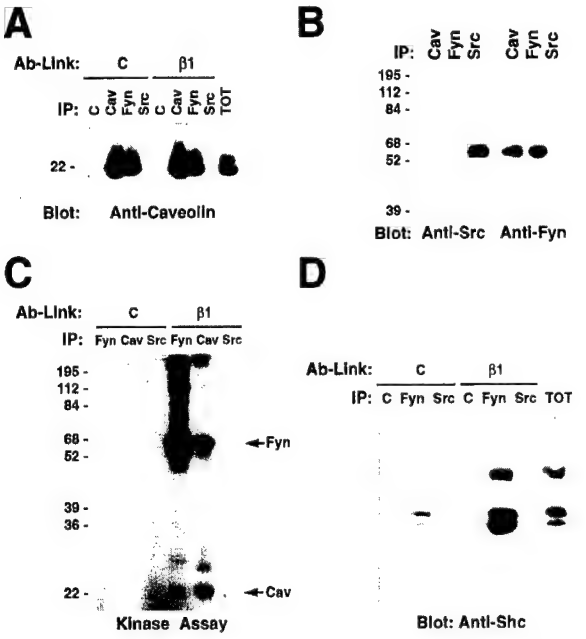


Figure 3. Caveolin-1 Combines with Fyn and thereby Shc

(A) WI-38 cells were incubated in suspension with W6.32 ("C") or 4B4 MAb (β1)-coated beads for 5 min, lysed in 1% Triton X-100, and immunoprecipitated with normal rabbit serum ("C") or affinity-purified rabbit antibody C13630 to caveolin-1 (Cav), 06–133 to the N terminus of Fyn (Fyn), and N-16 to the N terminus of Src (Src). Samples were subjected to immunoblotting with affinity-purified antibody N-20 to caveolin-1.

(B) WI-38 cells were lysed in 1% Triton X-100 and immunoprecipitated with affinity-purified antibody C13630 to caveolin-1 (Cav), 06–133 to Fyn (Fyn), or N-16 to Src (Src). Samples were subjected to immunoblotting with affinity-purified goat antibody sc-19G to the N terminus of Src or sc-16G to the N terminus of Fyn.

(C) WI-38 cells were cross-linked in suspension with W6.32 ("C") or 4B4 MAb (β1)-coated beads for 5 min, lysed in modified RIPA buffer, and immunoprecipitated with affinity-purified rabbit antibody C13630 to caveolin-1 (Cav), 06–133 to Fyn (Fyn), and N-16 to Src (Src). The samples were subjected to kinase assay and separated by SDS-PAGE. The gel was treated with alkali prior to autoradiography to remove radioactive phosphate bound to serine and threonine residues.

(D) WI-38 cells were cross-linked in suspension with W6.32 ("C") or 4B4 MAb (β1)-coated beads for 5 min, lysed in 1% Triton X-100, and immunoprecipitated with MAb W6.32 ("C"), MAb 15 (Fyn), or MAb GD11 (Src). Samples were subjected to immunoblotting with affinity-purified anti-Shc antibodies. A smaller aliquot of total lysate from unstimulated cells served as a control (TOT).

interacted with these signaling proteins. Coimmunoprecipitation experiments did not reveal any association of this fraction of caveolin-1 with the EGF receptor, Ras, and the Gsα subunit of heterotrimeric G proteins (unpublished). This further confirms the specificity of the association of caveolin-1 with both integrins and Fyn.

If caveolin-1-associated Fyn participates in integrin signaling, it should be activated by integrin ligation. To test this hypothesis, WI-38 fibroblasts were incubated with anti-β1 or control anti-MHC antibodies and subjected to immune complex kinase assay. The results indicated that ligation of β1 integrins activates total Fyn as well as the subset of Fyn associated with caveolin-1 (Figure 3C). The fraction of Fyn associated with caveolin-1 was also activated upon adhesion to fibronectin (not shown). Although upon cell adhesion to fibronectin, Src combines with autophosphorylated FAK and hence becomes active, we did not detect an activation of Src in cells cross-linked with anti-β1 antibodies (Figure 3C). Perhaps FAK is not fully activated under these experimental conditions or the fraction of Src that combines with autophosphorylated FAK is small in these cells.

Finally, if Fyn participates in the recruitment of Shc, integrin ligation should promote the association of Fyn with Shc. As shown in Figure 3D, coimmunoprecipitation analysis indicated that, upon integrin ligation, all three isoforms of Shc combine with Fyn. Taken together, these results suggest that Fyn mediates the recruitment of Shc in response to integrin ligation.

The SH3 Domain of Fyn Mediates Recruitment of Shc

In principle, Fyn could phosphorylate caveolin-1 or itself at a tyrosine residue able to interact with the SH2 or PTB domain of Shc. However, caveolin-1 is only weakly phosphorylated on tyrosine upon integrin ligation (not shown). In addition, neither caveolin-1 nor Fyn contain a consensus motif for binding to the SH2 or PTB domain of Shc. We thus examined if Fyn could recruit Shc in a phosphorylation-independent manner.

A previous study had shown that the SH3 domain of Fyn can interact with Shc in vitro (Weng et al., 1994). To examine the interaction of Fyn with Shc, Shc was immunoprecipitated from control and anti-β1 stimulated cells and probed by Far Western blotting with GST fusion proteins comprising the SH2, SH3, or both domains of Fyn. Figure 4A shows that the SH3 domain of Fyn, isolated or in the context of an SH3–SH2 fusion, directly binds to all three isoforms of Shc in vitro. Moreover, the binding does not require a modification of Shc induced by integrin ligation. In contrast, the SH2 domain of Fyn does not bind to Shc isolated from either control or anti-β1 stimulated cells. Thus, Fyn can directly interact with Shc in vitro; this interaction requires the SH3 domain of Fyn, but not tyrosine phosphorylation of either molecule.

The role of Fyn SH3 domain in vivo was examined by using fibroblasts derived from Fyn knockout mice. The $Fyn^{-/-}$ cells were transiently transfected with constructs encoding a wild-type or a mutant version of Fyn carrying a deletion of the SH3 domain and were then plated on poly-L-lysine or fibronectin. As shown in Figure 4B, adhesion to fibronectin induced association of the wild-type, but not SH3 mutant, Fyn with Shc. This indicates that the SH3 domain of Fyn is required for recruitment of Shc in vivo. The observation that the association of Fyn with Shc is not constitutive suggests that upon integrin-mediated activation, Fyn undergoes a conformational change that allows the ligand-binding surface of the SH3 domain to interact with Shc.

Fyn and Its SH3 Domain Are Required for Integrin-Dependent Shc–Ras–ERK Signaling

To examine if Fyn is essential for integrin signaling to ERK, wild-type, $Fyn^{-/-}$, and $Src^{-/-}$ fibroblasts were plated on poly-L-lysine or fibronectin and subjected to

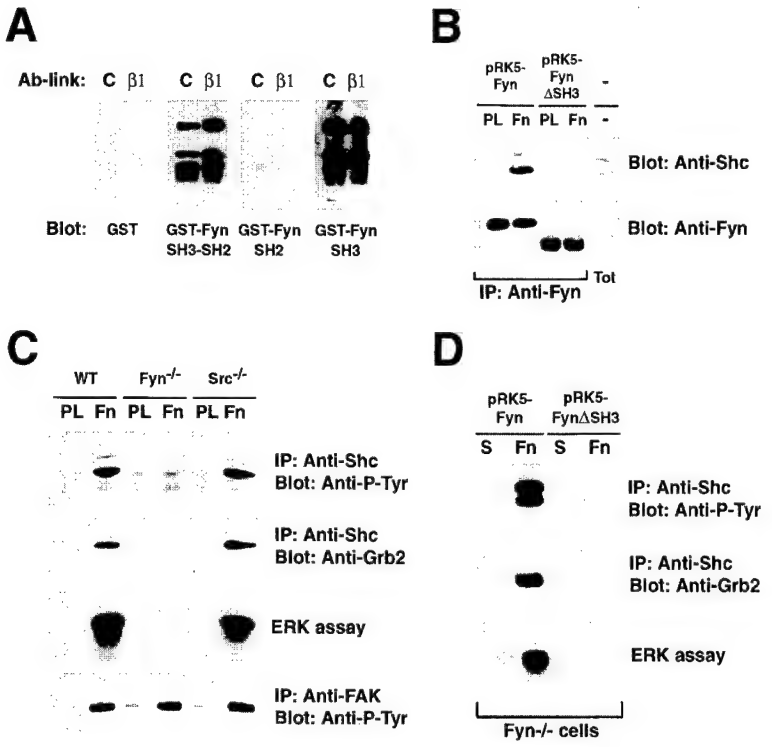


Figure 4. Role of the Fyn SH3 Domain in Recruitment of Shc and Activation of ERK

(A) WI-38 cells were cross-linked in suspension with W6.32 ("C") or 4B4 MAb (β1)-coated beads for 5 min, lysed in 1% Triton X-100, and immunoprecipitated with affinity-purified anti-Shc antibodies. Samples were subjected to Far Western analysis with control GST (GST) or GST fusion proteins comprising the SH3 and SH2 (GST-Fyn SH3-SH2), the SH2 (GST-Fyn SH2), or the SH3 domain of Fyn (GST-Fyn SH3). Bound fusion proteins were detected with anti-GST antibodies.

(B) *Fyn*$^{-/-}$ fibroblasts were transiently transfected with vectors encoding wild-type Fyn (pRK5-Fyn) or a mutant lacking the SH3 domain (pRK5-Fyn ΔSH3), detached, and plated on poly-L-lysine (PL) or fibronectin (Fn) for 20 min. After immunoprecipitation with anti-Fyn MAb 15, samples were subjected to immunoblotting with affinity-purified rabbit antibodies to Shc (top) or goat antibody sc-16G to Fyn (bottom). An aliquot of total lysate from cells transfected with empty vector (−) served as a control (TOT).

(C) Wild-type, *Fyn*$^{-/-}$, and *Src*$^{-/-}$ fibroblasts were detached and plated on poly-L-lysine (PL) or fibronectin (Fn) for 30 min. Equal amounts of total proteins were immunoprecipitated with polyclonal anti-Shc antibodies and probed with anti-P-Tyr MAb RC20-H (top panel) or anti-Grb2 polyclonal antibody (second panel). In addition, samples were immunoprecipitated with anti-ERK2 antibodies and subjected to immune complex kinase assay with MBP as a substrate (third panel) or immunoprecipitated with anti-FAK and probed with anti-P-Tyr MAb RC20-H (bottom panel).

(D) *Fyn*$^{-/-}$ fibroblasts were transiently transfected with vectors encoding wild-type Fyn (pRK5-Fyn) or a mutant lacking the SH3 domain (pRK5-Fyn ΔSH3), detached, and either kept in suspension (S) or plated on fibronectin (Fn) for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc polyclonal antibodies and probed with anti-P-Tyr MAb RC20-H (top panel) or anti-Grb2 polyclonal antibody (middle). The samples were also immunoprecipitated with anti-ERK2 antibodies and subjected to immune complex kinase assay with MBP as a substrate (bottom).

immunoprecipitation followed by immunoblotting or immune complex kinase assay. Adhesion of wild-type fibroblasts to fibronectin caused efficient tyrosine phosphorylation of Shc, association of Shc with Grb2, and activation of ERK. In contrast, adhesion of *Fyn*$^{-/-}$ cells to fibronectin failed to induce these events (Figure 4C). This indicates that Fyn is necessary for tyrosine phosphorylation of Shc, recruitment of Grb2, and activation of ERK in response to integrin ligation.

Whereas adhesion of the *Src*$^{-/-}$ fibroblasts to fibronectin caused efficient tyrosine phosphorylation of Shc and association of Shc with Grb2, it induced a somewhat reduced activation of ERK, consistent with a partial role of the FAK–Src complex in this process. Control experiments indicated that adhesion to fibronectin causes tyrosine phosphorylation of FAK to a similar extent in wild-type, *Fyn*$^{-/-}$, and *Src*$^{-/-}$ fibroblasts (Figure 4C). This latter observation is consistent with the notion that the major site of FAK phosphorylated in vivo is the autophosphorylation site, whereas the other sites can be phosphorylated by both Src and Fyn.

To examine the role of the Fyn SH3 domain in integrin signaling to ERK, the *Fyn*$^{-/-}$ cells were transiently transfected with a vector encoding wild-type or SH3 mutant Fyn and either kept in suspension or plated on fibronectin. As shown in Figure 4D, the introduction of wild-type Fyn restored integrin-mediated Shc signaling and activation of ERK in *Fyn*$^{-/-}$ cells. In contrast, transfection of the SH3 deletion mutant Fyn did not accomplish this effect (Figure 4D). These results provide direct evidence that Fyn and its SH3 domain are required for Shc signaling to ERK in response to integrin ligation.

Selective Activation of Fyn by Shc-Linked Integrins

To gain insight into the mechanism underlying the selective recruitment of Shc by a class of integrins, we examined the spectrum of integrins associated with caveolin-1. As shown in Figure 5A, all integrins tested, including α2β1 and α3β1, which are unable to recruit Shc, associated to a similar extent with caveolin-1. This observation suggests that the integrin association with caveolin-1 is necessary, but not sufficient, for recruitment of Shc.

To examine whether the integrin-specific step in the recruitment of Shc was upstream of Fyn, WI-38 cells were incubated in suspension with antibodies to various integrins and subjected to immunoprecipitation and immune complex kinase assay. Ligation of α1β1 and α5β1, which are functionally linked to Shc, caused activation of the fraction of Fyn associated with caveolin-1. By contrast, ligation of α2β1, α3β1, and α6β1, which are not linked to Shc, did not stimulate caveolin-1-associated Fyn (Figure 5B). Since all integrins examined combine with caveolin-1, and therefore presumably with Fyn,

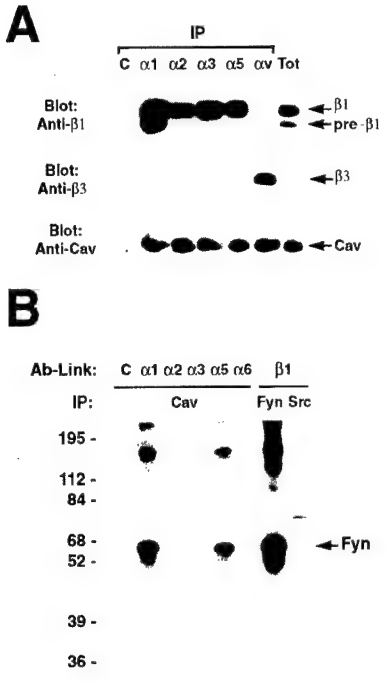


Figure 5. Several Integrins Associate with Caveolin-1, but Only Those Functionally Coupled to Shc Signaling Can Activate Fyn

(A) WI-38 cells were extracted with 1% Triton X-100 and immunoprecipitated with MAb W6.32 ("C"), TS2/7 (α1), P1E6 (α2), P1B5 (α3), P1D6 (α5), and LM609 (αv). The samples were subjected to immunoblotting with anti-β1 cytoplasmic domain serum (top), anti-β3 cytoplasmic domain serum (middle), and affinity-purified antibodies to the N terminus of caveolin-1 (bottom). An aliquot of the total extract was used as a control. The β3 subunit was detected in the total extract only upon prolonged exposure of the blot (not shown).

(B) WI-38 cells were cross-linked in suspension for 5 min with beads coated with MAb W6.32 ("C"), TS2/7 (α1), P1E6 (α2), P1B5 (α3), P1D6 (α5), GoH3 (α6), and 4B4 (β1). Lysates were immunoprecipitated with affinity-purified antibody C13630 to caveolin-1 (Cav), 06-133 to Fyn (Fyn), and N-16 to Src (Src) and subjected to kinase assay. The gel was treated with alkali prior to autoradiography.

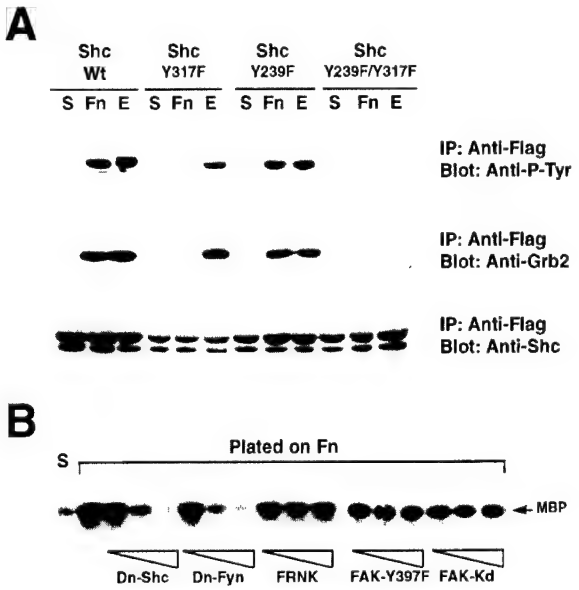


Figure 6. Phosphorylation of Shc Tyrosine 317 Mediates Activation of ERK in Response to Fibronectin

(A) 293-T cells were transiently transfected with vectors encoding Flag-tagged versions of Shc wild-type, Shc-Y317F, Shc-Y239F, or Shc-Y239/317F. The cells were detached and either kept in suspension (S) or plated on fibronectin (Fn) for 30 min. As a control, adherent cells were treated with 20 ng/ml EGF for 5 min (E). Cell lysates were immunoprecipitated with anti-Flag MAb M2 and subjected to immunoblotting with anti-P-Tyr MAb RC-20H (top) or anti-Grb2 antibodies (middle). The top blot was stripped and reprobed with anti-Shc antibodies (bottom).

(B) NIH-3T3 cells were transiently transfected with 1 μg of vector encoding HA-tagged ERK-2 alone or in combination with 5, 10, or 20 μg of vectors encoding Shc-Y317F (Dn-Shc), kinase-dead Fyn (Dn-Fyn), FRNK, FAK-Y397F, and kinase-dead FAK (Kd-FAK). The cells were held in suspension (S) or plated on dishes coated with 10 μg/ml fibronectin (Fn) for 30 min. Samples were immunoprecipitated with anti-HA MAb and subjected to kinase assay with MBP as a substrate. Expression levels were verified by immunoblotting total lysates.

these results suggest that an integrin-specific component functions upstream of Fyn to regulate the recruitment of Shc.

Shc Tyrosine 317 Mediates Recruitment of Grb2 and Activation of ERK in Response to Integrin Ligation

Shc contains two major tyrosine phosphorylation sites at positions 239 and 317 that can recruit the Grb2/SOS complex (van der Geer et al., 1996). To evaluate the relative roles of these sites in integrin signaling, 293-T cells were transiently transfected with vectors encoding Flag-tagged versions of Shc wild-type, Shc-Y239F, Shc-Y317F, and Shc-Y239F/Y317F and either plated on fibronectin or treated with EGF. The lysates were immunoprecipitated with anti-Flag, followed by immunoblotting with anti-P-Tyr or anti-Grb2 antibodies. Figure 6A shows that the single mutation at tyrosine 317 completely suppresses tyrosine phosphorylation of Shc and association of Shc with Grb2 in response to fibronectin. In contrast, only the combined phenylalanine substitutions at tyrosines 239 and 317 abolishes these events in cells stimulated with EGF. This indicates that tyrosine 317 is the major site in Shc that is phosphorylated and binds to Grb2 in response to integrin ligation.

The role of Shc tyrosine 317 in the activation of Ras–ERK signaling by integrins was also examined by a dominant negative approach. NIH-3T3 cells were transiently transfected with various doses of vectors encoding Shc-Y317F, kinase-dead Fyn, or three distinct mutant forms of FAK, all in combination with Flag-tagged ERK2. The FAK mutants included the noncatalytic C-terminal domain of the kinase (FRNK), which interferes with integrin-mediated activation of FAK and cell spreading; a version of the kinase with a phenylalanine substitution at tyrosine residue 397, which is unable to combine with Src-family kinases as well as PI 3-K; and a kinase-dead version. The transfectants were plated on fibronectin, and Flag-tagged ERK2 was immunoprecipitated and subjected to in vitro kinase assay. As shown in Figure 6B, both Shc-Y317F and kinase-dead Fyn caused a dose-dependent, dominant negative effect on ERK activation. In contrast, the three mutant forms of FAK exerted little

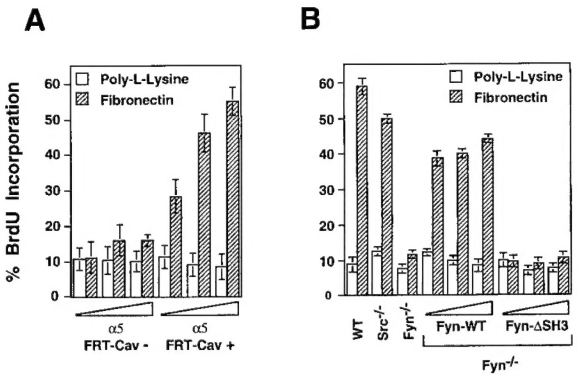


Figure 7. Requirement for Caveolin-1, Fyn, and the Fyn SH3 Domain during G1 Progression

(A) Parental and caveolin-1-expressing FRT cells were transiently transfected with 2.5, 5, and 10 μg of vector encoding the human α5 integrin subunit. The transfectants were sorted by panning on fibronectin, synchronized in G0 by growth factor deprivation, and plated on coverslips coated with poly-L-lysine (open bars) or fibronectin (stippled bars) in defined medium supplemented with EGF and BrdU. After 18 hr, the cells were fixed and stained with anti-BrdU MAb. Each column represents the mean ± SD from three independent experiments conducted in triplicate.

(B) Wild-type, *Src*$^{-/-}$, and *Fyn*$^{-/-}$ fibroblasts were synchronized in G0 by growth factor deprivation and plated on coverslips coated with poly-L-lysine (open bars) or fibronectin (stippled bars) in defined medium supplemented with PDGF and BrdU. After 16 hr, the cells were fixed and stained with anti-BrdU MAb. The *Fyn*$^{-/-}$ fibroblasts were also transiently transfected with 0.5 μg of vector encoding β-galactosidase in combination with 1.0, 2.5, and 5.0 μg of constructs encoding either wild-type Fyn (Fyn-WT) or SH3 mutant Fyn (Fyn-ΔSH3). The transfectants were synchronized in G0 by growth factor deprivation and plated on coverslips coated with poly-L-lysine (open bars) or fibronectin (stippled bars) in the presence of PDGF and BrdU. The number of transfected cells entering S phase was evaluated as described in Experimental Procedures. Each column represents the mean ± SD from three independent experiments conducted in triplicate.

or no effect. Control immunoblotting experiments confirmed that the expression level of the recombinant proteins was proportional to the amount of DNA transfected. These results indicate that fibronectin-induced phosphorylation of Shc at tyrosine 317 mediates recruitment of Grb2 and activation of Ras–ERK signaling.

Role of Caveolin-1 and Fyn in Cell Cycle Progression

The function of caveolin-1 in integrin-mediated cell cycle control was examined by using the caveolin-1-negative and -positive FRT cells. Upon introduction of an integrin α5 subunit, the cells were synchronized in G0 and plated on poly-L-lysine or fibronectin in the presence of EGF. Entry into the S phase was examined by 5′-Bromo-2′-deoxy-Uridine (BrdU) incorporation and anti-BrdU staining. Expression of α5β1 promoted entry in S phase of the caveolin-1-positive, but not -negative, FRT cells. The effect of α5β1 was dose dependent and required ligand binding because only a small percentage of both types of FRT cells entered S phase on poly-L-lysine (Figure 7A). These findings suggest that caveolin-1 is necessary to link α5β1 to the control of cell cycle.

To examine the role of Fyn in anchorage-dependent cell growth, wild-type, *Src*$^{-/-}$, and *Fyn*$^{-/-}$ fibroblasts were synchronized in G0 and plated on fibronectin or poly-L-lysine in the presence of PDGF. Adhesion to fibronectin, but not poly-L-lysine, induced entry into S of a similarly large fraction of wild-type and *Src*$^{-/-}$ cells. In contrast, only a modest percentage of *Fyn*$^{-/-}$ fibroblasts entered S on either fibronectin or poly-L-lysine (Figure 7B). This finding suggests that Fyn is required for progression through G1 and is consistent with the slow growth of *Fyn*$^{-/-}$ fibroblasts in culture.

Finally, we introduced in *Fyn*$^{-/-}$ cells wild-type or SH3 mutant Fyn in combination with the marker β-galactosidase. Entry of transfectants in S phase was evaluated by double staining with X-Gal and anti-BrdU antibodies. As shown in Figure 7B, wild-type, but not SH3 mutant, Fyn rescued the *Fyn*$^{-/-}$ fibroblasts from cell cycle arrest. The effect was dose dependent. The results indicate that Fyn and its SH3 domain are required for transit through G1. These findings provide evidence that the coupling of integrins to Shc–Ras–ERK signaling mediated by caveolin-1 and Fyn regulates cell cycle progression.

Discussion

The results of this study indicate that caveolin-1 functions as a membrane adaptor to couple integrins to the tyrosine kinase Fyn. Upon integrin ligation, Fyn is activated and recruits, via its SH3 domain, Shc. Upon phosphorylation at tyrosine 317, Shc combines with Grb2 and activates Ras–ERK signaling. These findings reveal an unexpected function of caveolin-1 and Fyn in integrin signaling and delineate a novel signaling pathway important for anchorage-dependent cell growth.

Caveolin-1 was independently identified as a substrate of v-Src (Glenney, 1989) and as a component of caveolae (Rothberg et al., 1992). While it is clear that caveolin-1 is necessary for the biogenesis of caveolae (Fra et al., 1995; Lipardi et al., 1998), the function of these structures, as distinct from rafts devoid of caveolin-1, is a matter of controversy. Cell fractionation studies have ascribed to caveolae a wide variety of signaling components and, by implication, functions (Okamoto et al., 1998). However, biochemical analysis of caveolae purified by an immunoisolation protocol does not support a signaling function for these structures, but leaves open the possibility that they function in intracellular transport (Stan et al., 1997). The results presented here reveal that a Triton X-100 soluble fraction of caveolin-1 that appears to be localized at extracellular matrix contacts plays a crucial role in integrin signaling. In light of this and the ability of v-Src to target adhesive junctions (Thomas and Brugge, 1997), it is not surprising that caveolin-1 was also identified as a substrate of v-Src.

Several observations suggest that the role of caveolin-1 in integrin signaling is specific and physiologically relevant. First, the Triton X-100 soluble fraction of caveolin-1 associates with integrins and Fyn, but not with a variety of signaling molecules that are thought to interact with caveolin-1 at caveolae (Okamoto et al., 1998). Accordingly, this fraction of caveolin-1 colocalizes with integrins at extracellular matrix contacts (unpublished).

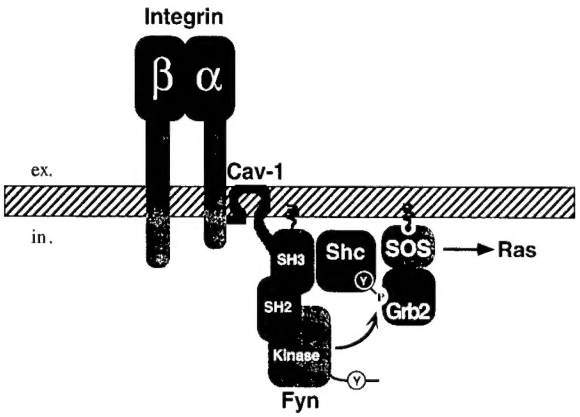


Figure 8. Model of Integrin-Mediated Recruitment of Shc and Activation of Ras

See text for further details.

Second, the same limited segment of integrin α subunit mediates both association with caveolin-1 and Shc signaling. Third, integrin ligation activates the fraction of Fyn associated with caveolin-1, establishing a further functional link between the three components. Finally, genetic evidence implies that both caveolin-1 and Fyn are necessary for activation of Shc–Ras–ERK signaling following integrin ligation.

The biochemical mechanisms underlying the association of integrins with caveolin-1 and of caveolin-1 with Fyn remain to be investigated. Because of its propensity to oligomerize, caveolin-1 is likely to contribute to the clustering of integrins at the cell surface. In principle, this mechanism may allow the activity of one integrin receptor to influence that of its neighbors (a phenomenon named activity spread), thereby increasing sensitivity to extracellular ligand (Bray et al., 1998). In order to understand integrin clusters in physiological and functional terms, it will be important to isolate them and examine the stoichiometry and spatial relationships of their essential elements. In addition, future studies will have to examine if integrins also interact with caveolin-2 and -3, as well as other Src-family kinases such as Yes, Lyn, and Lck. If proven, these mechanisms may expand the repertoire of signaling pathways affected by integrins.

Our results indicate that β1 and αv integrins recruit Shc and activate Ras signaling by the mechanism illustrated in Figure 8. We envision that integrin ligation activates a tyrosine phosphatase that in turn activates Fyn by desphosphorylating its C-terminal negative autoregulatory site. In support of this hypothesis, adhesion to fibronectin causes a transient activation of an integrin-associated enzyme capable of dephosphorylating the C-terminal tyrosine of Src-family kinases (F. Liu and F. G. G., unpublished). Once activated, Fyn combines with Shc, and this event requires an intact SH3 domain. Since in the inactive conformation of Src-family kinases the ligand-binding surface of the SH3 domain is occupied by the segment that links the kinase to the SH2 domain (Sicheri et al., 1997; Xu et al., 1997), it is likely that, upon integrin-mediated activation, Fyn undergoes a conformational change that exposes its SH3 domain, allowing it to interact with Shc. The ability of the isolated SH3 domain of Fyn to interact with Shc in vitro and the presence of SH3-binding motifs in the central domain of Shc support this model. Upon association with Fyn, Shc is phosphorylated at Tyr-317 and combines with Grb2. In contrast, EGF induces phosphorylation of tyrosine 239 and 317, which both contribute to the recruitment of Grb2. Many essential elements of the signal transduction mechanism described here are unexpected, most notably the function of caveolin-1 as a membrane adaptor, the role of Fyn SH3 domain in the recruitment of Shc, and the phosphorylation of Shc exclusively at tyrosine 317.

Since all integrins bind caveolin-1, and thereby Fyn, why does only a subset of them activate Fyn and recruit Shc? It is possible that the Shc-linked integrins associate with a required activator of Fyn, such as a phosphatase that dephosphorylates its C-terminal tyrosine. In addition, or instead, it may also be that the other integrins constitutively associate with a suppressor of Fyn, such as the kinase Csk that phosphorylates the same tyrosine residue (Thomas and Brugge, 1997). Finally, it cannot be excluded that the integrins not linked to Shc are able to constrain Fyn in an inactive conformation. Fortunately, with the essential elements identified, it will now be possible to determine if the integrin specificity of the Shc pathway arises from catalysis, constraint, or a combination of both.

Previous studies have indicated that, upon overexpression or association with elevated levels of Src, FAK can amplify the activation of Ras–ERK signaling in cells plated on fibronectin (Schlaepfer and Hunter, 1997; Schlaepfer et al., 1997). However, in normal fibroblasts, keratinocytes, and endothelial cells, integrin ligation activates Shc independently of FAK, and this event is both necessary and sufficient to activate the Ras–ERK pathway (Wary et al., 1996; Mainiero et al., 1997). Furthermore, the introduction of a dominant negative form of FAK or the deletion of the β1 subunit cytoplasmic domain suppresses activation of FAK without perturbing signaling to ERK (Lin et al., 1997a). The results of this study support the model that integrins are linked to the Ras–ERK pathway by Shc, and this occurs independently of FAK and Src. First, although the activation of FAK is mediated by the integrin β subunit cytoplasmic domain, a limited segment of the integrin α subunit is sufficient for both recruitment of Shc and activation of ERK. Second, adhesion to fibronectin causes tyrosine phosphorylation of Shc, association of Shc with Grb2, and activation of ERK in wild-type and *Src*$^{-/-}$ fibroblasts, but not in *Fyn*$^{-/-}$ fibroblasts. Consistent with these findings, the expression of recombinant wild-type, but not SH3 mutant, Fyn rescues signaling to ERK in *Fyn*$^{-/-}$ fibroblasts. Finally, dominant negative versions of Fyn and Shc suppress ERK activation in normal fibroblasts plated on fibronectin, while three dominant negative forms of FAK exert little or no effect. Thus, it appears that, at least in the normal cells we have examined, integrins activate Ras–ERK signaling via the pathway involving the integrin α subunit, caveolin-1, Fyn, and Shc.

What is the biological significance of integrin-mediated Shc signaling? The observation that the Mos–ERK cascade, which regulates *Xenopus* oocyte maturation,

is intrinsically ultrasensitive provides a biochemical rationale for the existence of a threshold in MAPK cascades and the all-or-none character of cell fate switches that they regulate (Ferrell and Machleder, 1998). In light of this, the simplest hypothesis is that a combined stimulation of Ras by Shc-linked integrins and growth factor receptors is required to activate ERK beyond the threshold level required for immediate-early gene expression. While other mechanisms exist to ensure that cells that have lost contact with the extracellular matrix do not respond to growth factor stimulation (Lin et al., 1997b; Renshaw et al., 1997), the principle here proposed is likely to explain the distinct effects of different extracellular matrices on the cell cycle. When cell adhesion is predominantly mediated by Shc-linked integrins, growth factor stimulation results in cell proliferation. In contrast, when adhesion is mediated by other integrins, cells exit from the cell cycle despite the presence of growth factors. The cell cycle defects observed in mice carrying a targeted deletion of the integrin β4 subunit cytoplasmic domain provide evidence that the mechanism illustrated here operates also in vivo (Murgia et al., 1998). Finally, since most dominant oncogenes constitutively activate the Ras–ERK pathway and most tumor suppressors inhibit cell cycle progression by acting downstream of this pathway, our model also explains in a natural way why neoplastic cells display anchorage-independent growth.

Experimental Procedures

Antibodies and Extracellular Matrix Proteins

MAbs anti-integrin, W6.32, 4E3, M2, and RC20-H, affinity-purified antibodies to Grb2 and ERK2, and rabbit antisera to the C termini of integrin β1 and FAK were as described previously (Giancotti and Ruoslahti, 1990; Wary et al., 1996). MAbs 15 (to Fyn amino acids 85–206) and PG-797 (to the Shc SH2 domain), affinity-purified rabbit antibodies N-20 to the caveolin-1 N terminus, sc-16 to Fyn residues 28–48 and sc-16 to Src residues 3–18, and affinity-purified goat antibodies sc-16G to Fyn residues 28–48 and sc-19G to Src residues 3–18 were from Santa Cruz Biotechnology. MAb GD11 to Src and rabbit polyclonal antibodies 06–133 to Fyn residues 35–51 were from Upstate Biotechnology. MAb CO60 and affinity-purified rabbit antibodies C13630, both to the N-terminal domain of caveolin-1, were from Transduction Laboratories. Anti-phospho-ERK antibodies were from NEB. MAb P1G12 to CD44 was from W. Carter (Fred Hutchinson Cancer Research Center). Affinity-purified antibodies to a GST fusion protein comprising the SH2 domain of Shc and to GST were generated in our laboratory. Human fibronectin was from GIBCO-BRL.

Cell Lines, Constructs, and Transfections

WI-38 and NIH-3T3 cells were from the ATCC. Immortalized 3T3 lines derived from wild-type, *Fyn*$^{-/-}$, and *Src*$^{-/-}$ mice were provided by P. Soriano (Fred Hutchinson Cancer Research Center). 293-T cells were provided by D. Levy (N.Y.U. School of Medicine). The caveolin-1-negative FRT cells and the caveolin-1-expressing FRT-Cav-13 and FRT-Cav-22 cells were described previously (Lipardi et al., 1998).

All eukaryotic expression vectors were based on the CMV promoter. Vectors encoding the single chain tailless α1 subunit (A: amino acids 538–1138), the IL2-R α chain, HA-tagged Erk-2, β-galactosidase, Flag-tagged Shc, and Flag-tagged Shc Y317F were described previously (Wary et al., 1996). Vectors encoding wild-type and kinase-dead Fyn were obtained from J. Sap (N.Y.U. School of Medicine). To generate the SH3 domain deletion mutant, pRK5-Fyn was cut with SpII, filled in with G+T, and flushed with Mung bean exonuclease. After digestion with SalI and HincII, the 1.4 Kb fragment encoding the SH2, kinase, and C-terminal domain of Fyn was ligated to the 4.9 Kb fragment containing the vector and N-terminal sequences of Fyn. Transient transfection in 293-T cells followed by immune complex kinase assay indicated that the SH3 domain mutant Fyn had a kinase activity similar to that of wild-type Fyn. Vectors encoding FRNK, kinase-dead FAK, and FAK Y397F were provided by J.-L. Guan (Cornell University School of Veterinary Medicine). The human α5 subunit cDNA (Giancotti and Ruoslahti, 1990) was subcloned in the expression vector pCB7. Eukaryotic vectors encoding Flag-tagged Shc Y239F and Y239/317F and the α1/IL2-R α chimeras (B: residues 538–1114 of α1 fused to 231–262 of IL2-R α; C: 1–229 of IL2-R α fused to 1059–1170 of α1) and bacterial vectors encoding GST fusion proteins comprising the SH3-SH2 (residues 82–250), SH3 (82–145), and SH2 (146–250) domain of Fyn were generated by two-step PCR and verified by dideoxy sequencing.

The FRT, FRT–Cav-13, and FRT–Cav-22 cells express very low levels of α5β1 and do not attach to fibronectin in short-term adhesion assays. They were thus transiently transfected with pCB7-α5. Cells expressing α5β1 at their surface were isolated by panning on fibronectin and examined by FACS analysis prior to subsequent analysis. All cells were transiently transfected with Lipofectamine (GIBCO-BRL) and allowed to recover in complete medium prior to growth factor starvation.

Biochemical Methods

Prior to biochemical analysis, cells were serum starved for 36 hr, detached with 0.02% EDTA, and kept in suspension in serum-free medium for 30 min. To ligate integrins at the cell surface with antibodies, 2×10^7 cells were resuspended in 0.2 ml of DMEM, mixed with an equal volume of DMEM containing 50 μl of polystyrene latex beads that had been coated with 20 μg of purified control or anti-integrin MAbs as previously described (Mainiero et al., 1995), and incubated at 37°C for the indicated times. For coimmunoprecipitation of integrins with Shc, an alternative protocol was also used. The cells were resuspended in 0.2 ml DMEM, incubated on ice with 10 μg of purified control or anti-integrin MAbs for 40 min, washed once at 4°C, and then incubated at 37°C with 5 μg of affinity-purified rabbit anti-mouse IgGs. After adding 1 ml of lysis buffer and 5 μg of anti-integrin MAb, the extracts were incubated on ice for 1 hr and finally clarified prior to recovering the immune complexes with Sepharose Protein G. To examine signaling in response to cell adhesion to the extracellular matrix, the cells were either kept in suspension or replated at subconfluent densities on dishes coated with 5 μg/ml poly-L-lysine or 10 μg/ml fibronectin.

For immunoprecipitation followed by immunoblotting, cells were extracted for 1 hr at 4°C with 1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, and protease and phosphatase inhibitors. For immunoprecipitation of integrins, lysis buffer included 1 mM $CaCl_2$ and 1 mM $MgCl_2$, but no EDTA. Immunoprecipitation, immunoblotting, and kinase assays were performed essentially as previously described (Mainiero et al., 1995; Wary et al., 1996). For alkali treatment, gels were incubated in 1 M KOH for 2 hr at 60°C and neutralized prior to autoradiography. For Far Western analysis, blots were saturated with 5% nonfat dry milk and 2% BSA and incubated with 2 μg/ml GST fusion proteins followed by 0.5 μg/ml rabbit anti-GST antibodies and peroxidase-conjugated protein A.

Analysis of Cell Cycle Progression

FRT and FRT–Cav-13 cells were transfected with various doses of pCB7-α5, synchronized in G0 by growth factor deprivation, and panned on fibronectin. FACS analysis was used to verify that the level of expression of α5β1 at their surface was comparable and proportional to the amount of DNA introduced. Cells were then plated on microtiter wells coated with 5 μg/ml poly-L-lysine or 10 μg/ml fibronectin in defined medium (Coon's F12 supplemented + ITS) containing 20 ng/ml EGF and 10 μM BrdU. After 18 hr, cells were stained with anti-BrdU MAb and AP-conjugated anti-mouse IgGs (Boehringer).

Wild-type, *Fyn*$^{-/-}$, and *Src*$^{-/-}$ cells were analyzed as described above except that the medium consisted of DMEM + ITS containing 20 ng/ml PDGF. For cell cycle rescue experiments, *Fyn*$^{-/-}$ cells were transiently transfected with vector encoding β-galactosidase in

combination with various doses of constructs encoding either wild-type or SH3 mutant Fyn. Cells were fixed and stained with X-Gal followed by anti-BrdU MAb and AP-conjugated anti-mouse IgGs. The percentage of X-Gal-positive cells that had incorporated BrdU was evaluated microscopically after light counterstaining with hematoxylin.

Acknowledgments

We are indebted to P. Soriano for the *Src* $^{-/-}$ and *Fyn* $^{-/-}$ fibroblasts. We thank L. Nitsch for the parental FRT cells, and W. Carter, D. Cheresh, J.-L. Guan, E. Marcantonio, J. Sap, and G. Tarone for antibodies and constructs. We are grateful to C. Blobel, B. Gumbiner, E. Marcantonio, M. Resh, J. Rothman, and members of the Giancotti laboratory for discussions and comments on the manuscript. This work was supported by DAMD grant 17-94-J4306 (F. G. G.), NIH grants CA58976 (F. G. G.) and P30 CA08748 (M. S. K. C. C.), and a fellowship from the American Italian Cancer Foundation. F. G. G. is an Established Investigator of the AHA.

Received March 25, 1998; revised July 23, 1998.

References

Bray, D., Levin, M.D., and Morton-Firth, C.J. (1998). Receptor clustering as a cellular mechanism to control sensitivity. Nature *393*, 85–88.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu. Rev. Dev. Biol. *12*, 463–519.

Chen, W.-T., Hasegawa, E., Hasegawa, T., Weinstock, C., and Yamada, K.M. (1985). Development of cell surface linkage complexes in cultured fibroblasts. J. Cell Biol. *100*, 1103–1114.

Clark, E.A., and Hynes, R.O. (1997). Meeting report: 1997 Keystone symposium on signal transduction by cell adhesion receptors. Biochim. Biophys. Acta *1333*, R9-R16.

Ferrell, J.E., and Machleder, E.M. (1998). The biochemical basis of an all-or-none cell fate switch in Xenopus oocytes. Science *280*, 895–898.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. (1995). De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. Proc. Natl. Acad. Sci. USA *92*, 8655–8659.

Giancotti, F.G. (1997). Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. *9*, 691–700.

Giancotti, F.G., and Mainiero, F. (1994). Integrin-mediated adhesion and signaling in tumorigenesis. Biochim. Biophys. Acta *1198*, 47–64.

Giancotti, F.G., and Ruoslahti, E. (1990). Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell *60*, 849–859.

Glenney, J.R. (1989). Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. J. Biol. Chem. *264*, 20163–20166.

Harder, T., and Simons, K. (1997). Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr. Opin. Cell Biol. *9*, 534–542.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549–554.

Koleske, A.J., Baltimore, D., and Lisanti, M.P. (1995). Reduction of caveolin and caveolae in oncogenically transformed cells. Proc. Natl. Acad. Sci. USA *92*, 1381–1385.

Lin, T.H., Aplin, A.E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R.L. (1997a). Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. J. Cell Biol. *136*, 1385–1395.

Lin, T.H., Chen, Q., Howe, A., and Juliano, R.L. (1997b). Cell anchorage permits efficient signal transduction between Ras and its downstream kinases. J. Biol. Chem. *272*, 8849–8852.

Lipardi, C., Mora, R., Colomer, V., Paladino, S., Nitsch, L., Rodriguez-Boulan, E., and Zurzolo, C. (1998). Caveolin transfection results in caveolae formation but not apical sorting of glycosylphosphatidylinositol (GPI)-anchored proteins in epithelial cells. J. Cell Biol. *140*, 617–626.

Mainiero, F., Pepe, A., Wary, K.K., Spinardi, L., Mohammadi, M., Schlessinger, J., and Giancotti, F.G. (1995). Signal transduction by the $\alpha6\beta4$ integrin: distinct $\beta4$ subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. EMBO J. *14*, 4470–4481.

Mainiero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumenberg, M., Westwick, J.K., Der, C.J., and Giancotti, F.G. (1997). The coupling of $\alpha6\beta4$ integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. EMBO J. *16*, 2365–2375.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

Monier, S., Parton, R.G., Vogel, F., Henske, A., and Kurzchalia, T. (1995). VIP21-caveolin, a membrane protein constituent of the caveolar coat, forms high molecular mass oligomers in vivo and in vitro. Mol. Biol. Cell *6*, 911–927.

Murgia, C., Blaikie, P., Kim, N., Dans, M., Petrie, H.T., and Giancotti, F.G. (1998). Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin $\beta4$ cytoplasmic domain. EMBO J. *17*, 3940–3951.

Okamoto, T., Schlegel, A., Scherer, P.E., and Lisanti, M.P. (1998). Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J. Biol. Chem. *273*, 5419–5422.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075–2080.

Plantefaber, L.C., and Hynes, R.O. (1989). Changes in integrin expression on oncogenically transformed cells. Cell *56*, 281–290.

Renshaw, M.W., Ren, X.-D., and Schwartz, M.A. (1997). Growth factor activation of MAP kinase requires cell adhesion. EMBO J. *16*, 5592–5599.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.-S., Glenney, J.R., and Anderson, R.G.W. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673–682.

Ruoslahti, E., and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238*, 491–497.

Schlaepfer, D.D., and Hunter, T. (1997). Focal adhesion kinase overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interaction with and activation of c-Src. J. Biol. Chem. *272*, 13189–13195.

Schlaepfer, D.D., Broome, M.A., and Hunter, T. (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130^{cas}, and Nck adaptor proteins. Mol. Cell. Biol. *17*, 1702–1713.

Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature *385*, 602–609.

Stan, R.-V., Roberts, W.G., Predescu, D., Ihida, K., Saucan, L., Ghitescu, L., and Palade, G.E. (1997). Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Mol. Biol. Cell *8*, 595–605.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. *13*, 513–609.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. *8*, 205–215.

van der Geer, P., Wiley, S., Gish, G.D., and Pawson T. (1996). The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediated protein-protein interactions. Curr. Biol. *6*, 1435–1444.

Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E., and Giancotti, F.G. (1996). The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell *87*, 733–743.

Weng, Z., Thomas, S.M., Rickles, R.J., Taylor, J.A., Brauer, A.W., Seidel-Dugan, C., Michael, W.M., Dreyfuss, G., and Brugge, J.S. (1994). Identification of Src, Fyn, and Lyn SH3-binding proteins: implications for a function of SH3 domains. Mol. Cell. Biol. *14*, 4509–4521.

Xu, W., Harrison, S.C., and Eck, M.J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595–602.